CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of Serial No. 09/066,652, filed April 27, 1998, which is a continuation-in-part of Serial No. 09/004,606, filed January 8, 1998, which is a continuation-in-part of Serial No. 08/888,057 which is a continuation-in-part of Serial No. 08/781,752, the contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to cloning procedures in which cell nuclei derived from differentiated fetal or adult bovine cells, which include non-serum starved differentiated fetal or adult bovine cells, are transplanted into enucleated oocytes of the same species as the donor nuclei. The nuclei are reprogrammed to direct the development of cloned embryos, which can then be transferred into recipient females to produce fetuses and offspring, or used to produce cultured inner cell mass cells (CICM). Fetuses and animals derived from a single clonal line offer a safe and genetically modifiable source of transplantation tissue. The cloned embryos can also be combined with fertilized embryos to produce chimeric embryos, fetuses and/or offspring.

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All of the above publications, patent applications and patents are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Genetic modification of ungulates such as cattle or pigs could be useful in increasing the efficiency of meat and/or milk production and generate a useful source of cells and tissues for xenotransplantation. An ideal system for producing transgenic animals for such applications would be highly efficient and use small numbers of recipient animals to produce transgenics. It would allow the insertion of a transgene or the detection of a specific DNA sequence, into a specific genotype. The insertion or

deletion would preferably be into a predetermined site, e.g., effected via homologous recombination, which insertion or deletion would confer high expression and not affect general viability and productivity of the animal. Furthermore, the identification of a locus for insertion would allow multiple lines to be produced and crossed to produce homozygotes and new genetic background could easily be added to the transgenic line by the production of new transgenics at any time. Therefore, the ideal system would likely require the transfection and selection of cells that could be easily grown in culture yet retain the potency to form germ cells and pass the gene to subsequent generations.

Various methods have been utilized in an attempt to genetically modify ungulates such as cattle so as to introduce superior agricultural qualities including in particular pronuclear microinjection. However, a significant limitation of pronuclear microinjection is that the gene insertion site is inherently random. This typically results in variations in expression levels and several transgenic lines must be produced to obtain one line with appropriate levels of expression to be useful.

Because integration is random, it is advantageous that a line of transgenic animals be started from one founder animal, to avoid difficulties in monitoring zygosity and potential difficulties that might occur with interactions among multiple insertion sites.[8] Furthermore, starting a transgenic line from one hemizygous animal with a random insert would require breeding several generations and significant time for introgression of the transgene into the population before breeding and testing homozygotes if inbreeding is to be avoided.[8] Even without concern for inbreeding, it would take 6.5 years before reproduction could be tested in homozygous animals.[26] Finally, the quality of the genetics of a monozygous transgenic line would lag behind that of the general population because of the reduced population within which to select future generations of transgenic animals and the difficulty of bringing new genetics into a population in which the transgene is fixed.

A second limitation of the pronuclear microinjection procedure is its efficiency; which ranges from 0.34 to 2.63% of the gene-injected embryos developing into transgenic animals and a fraction of these appropriately ex-

pressing the gene.[24] This inefficiency results in a high cost of producing transgenic cattle because of the large number of recipients needed and, more importantly, unpredictability in the genetic background into which the gene is inserted because of the large number of embryos needed for microinjection. For agricultural purposes, a high quality genetic background is essential, therefore, long-term backcrossing strategies must be used with pronuclear microinjection. Thus, the ability to clone, or to make numerous identical genetic copies, of an animal comprising a desired genetic modification would be advantageous.

Another such system for producing transgenic animals has been developed and widely used in the mouse and involves the use of embryonic stem (ES) cells.

Embryonic stem cells in mice have enabled researchers to select for transgenic cells and perform gene targeting. This allows more genetic engineering than is possible with other transgenic techniques. Mouse ES cells are relatively easy to grow as colonies *in vitro*. The cells can be transfected by standard procedures and transgenic cells clonally selected by antibiotic resistance.[9] Furthermore, the efficiency of this process is such that sufficient trans-

genic colonies (hundreds to thousands) can be produced to allow a second selection for homologous recombinants.[9] Mouse ES cells can then be combined with a normal host embryo and, because they retain their potency, can develop into all the tissues in the resulting chimeric animal, including the germ cells. The transgenic modification can then be transmitted to subsequent generations.

Methods for deriving embryonic stem (ES) cell lines *in vitro* from early preimplantation mouse embryos are well known.[10, 18] ES cells can be passaged in an undifferentiated state, provided that a feeder layer of fibroblast cells[10] or a differentiation inhibiting source[28] is present.

ES cells have been previously reported to possess numerous applications. For example, it has been reported that ES cells can be used as an *in vitro* model for differentiation, especially for the study of genes which are involved in the regulation of early development. Mouse ES cells can give rise to germline chimeras when introduced into preimplantation mouse embryos, thus demonstrating their pluripotency.[2]

In view of their ability to transfer their genome to the next generation, ES cells have potential utility for germline manipulation of livestock animals by using ES cells with or without a desired genetic modification. Some research groups have reported the isolation of purportedly pluripotent embryonic cell lines. For example, Notarianni, *et al.*[20] reports the establishment of purportedly stable, pluripotent cell lines from pig and sheep blastocysts which exhibit some morphological and growth characteristics similar to that of cells in primary cultures of inner cell masses isolated immunosurgically from sheep blastocysts. Also, Notarianni, *et al.*[19] discloses maintenance and differentiation in culture of putative pluripotential embryonic cell lines from pig blastocysts. Gerfen, *et al.*[13] discloses the isolation of embryonic cell lines from porcine blastocysts. These cells are stably maintained without mouse embryonic fibroblast feeder layers and reportedly differentiate into several different cell types during culture.

Further, Saito, *et al.*[25] reports cultured, bovine embryonic stem cell-like cell lines which survived three passages, but were lost after the fourth passage. Handy-

side, *et al.*[15] discloses culturing of immunosurgically isolated inner cell masses of sheep embryos under conditions which allow for the isolation of mouse ES cell lines derived from mouse ICMs. Handyside, *et al.* also reports that under such conditions, the sheep ICMs attach, spread, and develop areas of both ES cell-like and endoderm-like cells, but that after prolonged culture only endoderm-like cells are evident.

Recently, Cherny, *et al.*[5] reported purportedly pluripotent bovine primordial germ cell-derived cell lines maintained in long-term culture. These cells, after approximately seven days in culture, produced ES-like colonies which stained positive for alkaline phosphatase (AP), exhibited the ability to form embryoid bodies, and spontaneously differentiated into at least two different cell types. These cells also reportedly expressed mRNA for the transcription factors OCT4, OCT6 and HES1, a pattern of homeobox genes which is believed to be expressed by ES cells exclusively.

Also recently, Campbell, *et al.*[4] reported the production of live lambs following nuclear transfer of cultured

embryonic disc (ED) cells from day nine ovine embryos cultured under conditions which promote the isolation of ES cell lines in the mouse. The authors concluded that ED cells from day nine bovine embryos are totipotent by nuclear transfer and that totipotency is maintained in culture.

Van Stekelenburg-Hamers, *et al.*[32] reported the isolation and characterization of purportedly permanent cell lines from inner cell mass cells of bovine blastocysts. The authors isolated and cultured ICMs from 8 or 9 day bovine blastocysts under different conditions to determine which feeder cells and culture media are most efficient in supporting the attachment and outgrowth of bovine ICM cells. They concluded that the attachment and outgrowth of cultured ICM cells is enhanced by the use of STO (mouse fibroblast) feeder cells (instead of bovine uterus epithelial cells) and by the use of charcoal-stripped serum (rather than normal serum) to supplement the culture medium. Van Stekelenburg, *et al.* reported, however, that their cell lines resembled epithelial cells more than pluripotent ICM cells.

Smith, *et al.*[36], Evans, *et al.*[35], and Wheeler, *et al.*[37] report the isolation, selection and propagation of animal stem cells which purportedly may be used to obtain transgenic animals. Evans, *et al.* also reported the derivation of purportedly pluripotent embryonic stem cells from porcine and bovine species which assertedly are useful for the production of transgenic animals. Further, Wheeler, *et al.* disclosed embryonic stem cells which are assertedly useful for the manufacture of chimeric and transgenic ungulates.

Alternatively, ES cells from a transgenic embryo could be used in nuclear transplantation. The use of ungulate inner cell mass (ICM) cells for nuclear transplantation has also been reported. In the case of livestock animals, e.g., ungulates, nuclei from like preimplantation livestock embryos support the development of enucleated oocytes to term.[16,29] This is in contrast to nuclei from mouse embryos which beyond the eight-cell stage after transfer reportedly do not support the development of enucleated oocytes.[6] Therefore, ES cells from livestock animals are highly desirable because they may provide a potential source of

totipotent donor nuclei, genetically manipulated or otherwise, for nuclear transfer procedures.

Collas, *et al.*[7] discloses nuclear transplantation of bovine ICMs by microinjection of the lysed donor cells into enucleated mature oocytes. Collas, *et al.* disclosed culturing of embryos *in vitro* for seven days to produce fifteen blastocysts which, upon transferral into bovine recipients, resulted in four pregnancies and two births. Also, Keefer, *et al.*[16] disclosed the use of bovine ICM cells as donor nuclei in nuclear transfer procedures, to produce blastocysts which, upon transplantation into bovine recipients, resulted in several live offspring. Further, Sims, *et al.*[27] disclosed the production of calves by transfer of nuclei from short-term *in vitro* cultured bovine ICM cells into enucleated mature oocytes.

Thus, based on the foregoing, it is evident that many groups have attempted to produce ES cell lines, e.g., because of their potential application in the production of cloned or transgenic embryos, nuclear transplantation, and for producing differentiated cells *in vitro*.

However, embryonic stem cell lines and other embryonic cell lines must be maintained in an undifferentiated state that requires feeder layers and/or the addition of cytokines to media. Even if these precautions are followed, these cells often undergo spontaneous differentiation and cannot be used to produce transgenic offspring by currently available methods. Also, some embryonic cell lines have to be propagated in a way that is not conducive to gene targeting procedures. Thus, genetic modification using differentiated cells for transgenic and nuclear transfer techniques would be advantageous.

The production of live lambs following nuclear transfer of cultured embryonic disc cells has also been reported.[4] Still further, the use of bovine pluripotent embryonic cells in nuclear transfer and the production of chimeric fetuses has been reported[7,31] Collas, *et al.*[7] demonstrated that granulosa cells (adult cells) could be used in a bovine cloning procedure to produce embryos. However, there was no demonstration of development past early embryonic stages (blastocyst stage). Also, granulosa cells are not easily cultured and are only obtainable from females. Collas, *et al.*[7] did not attempt to propagate the granulosa

cells in culture or try to genetically modify those cells. Wilmut, *et al.*[34] produced nuclear transfer sheep offspring derived from fetal fibroblast cells, and one offspring from a cell derived from an adult sheep.

Cloning sheep cells has been easier in comparison with cells of other species. This phenomenon is illustrated by the following table:

SPECIES (from hardest to easiest to clone)	CELL TYPE CLONED	OFFSPRING PRODUCED
Pig (Prather, *Biol. Report*, 41:414-418, 1989)	2 and 4 cell stage embryo	yes
Pig (Prather, *Id.*, 1989;	greater than 4 cell stage	no
Mouse (Cheong, *et al.*, *Biol. Reprod.*, 48:958-963, 1993)	2,4 and 8 cell stage embryo	yes
Mouse (Tsunoda, *et al.*, *J. Reprod. Fertil.*, 98:537-540, 1993)	greater than 8 cell stage	no
Cattle (Keefer, *et al.*, *Biol. Reprod.*, 50:935-939, 1994)	64 to 128 cell stage (ICM)	yes
Cattle (Stice, *et al.*, *Biol. Repro.*, 54:100-110, 1996)	embryonic cell line from ICM	no
Sheep (Campbell, *et al.*, *Nature*, 380:64-66, 1996)	embryonic cell line from ICM	yes
Sheep (Wilmut, *et al.*, *BARC Symposia*,20:145-150, 1997)	fetal and adult cells	yes

However, there exist problems in the area of producing transgenic cows. By current methods, heterologous DNA is introduced into either early embryos or embryonic cell lines that differentiate into various cell types in the fetus and eventually develop into a transgenic animal. One limitation is that many early embryos are required to produce one transgenic animal and, thus, this procedure is very inefficient. Also, there is no simple and efficient method of selecting for a transgenic embryo before going through the time and expense of putting the embryos into surrogate females. In addition, gene targeting techniques cannot be easily accomplished with early embryo transgenic procedures.

Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of cloning ungulates such as cows and pigs. A consistent and efficient source of cloned ungulates, e.g., cows or pigs, would provide the potential for the cells and tissues of such cloned ungulates to have widespread use in xenotransplantation.

In this regard, transplantation of tissues and organs has applications in the treatment of various diseases,

e.g., diabetes, cardiovascular diseases, autoimmune diseases, kidney disease, various cancers, neurological disorders and many others.

One particular neurological disease that may be treated by transplanted tissue or cells comprises Parkinson's disease. For instance, symptoms of Parkinson's disease can be improved by transplantation of human fetal dopamine cells into the putamen of Parkinsonian patients. However, the supply of suitable human donor tissue is limited and variable. Accordingly, an alternative non-human source of tissue, i.e., xenotransplanted tissue, would be valuable. Although xenografts from outbred animals have raised concerns about latent viruses, animals derived from a single clonal line offer a safe and genetically modifiable source of transplantable tissue.

Fetal tissue transplantation is used worldwide to alleviate symptoms of Parkinson's disease (41-48). A major problem of this emerging therapy is limited supply of the human fetal tissue. To address this shortcoming, others have studied transplanted non-human fetal tissue in the 6-hydroxydopamine-lesioned (6-OHDA) rat model of Parkinson's disease (hemiparkinsonian rat). Transplantation of

porcine, rabbit, and mouse ventral mesencephalon into hemiparkinsonian rats revealed that dopamine cells survive in such xenografts (49-52). About 100 surviving porcine dopamine cells are required to improve motor deficits by at least 50% in this animal model (53). Recently, fetal pig neural cells have been shown to survive in an immunosuppressed parkinsonian patient (54).

Cloned ungulate fetal tissue, in particular cloned pig or bovine fetal tissue, would provide a convenient and alternative source of tissue for neural xenotransplantation. Although pig tissue has been used in previous xenotransplantation studies (49-54), *in vitro* embryo production and cloning technologies are now more advanced in cattle. Prior to the present invention, methods only existed for producing early porcine embryos by cloning. This prohibited attempts to produce large numbers of cloned transgenic fetuses (Prather, R.S., Sims, M.N., & First, N.L. Nuclear transplantation in early pig embryos. *Biol. Reprod.* 41, 414-418 (1989)). However, traditional procedures for producing transgenic pigs are inefficient. Less than 1% of porcine embryos can be made transgenic and

gene targeting (Pursel, V.G. & Rexroad, C.E. Jr. Status of research with transgenic farm animals. *J. Animal Sci.* 71 (Suppl. 3), 10-19 (1993)). In this regard, copending Appl. Serial No. 08/888,057, filed on July 3, 1997, provides an improved method for producing cloned pigs and embryos which should alleviate the problems of the previous techniques. In particular, this application describes a method for cloning pigs, which optionally may be transgenic, that should obviate the inefficiencies of previous methods by nuclear transfer using differentiated cells as the donor cells, e.g., fibroblasts. This application is herein incorporated by reference in its entirety.

Improvements in the efficiency and safety of eventual xenotransplantation treatment for Parkinson's disease may be realized through animal cloning and transgenic technologies. First, animal cloning technology may be capable of producing a continuous supply of fetal neuronal tissue having identical genetic background. Since multiple fetuses are required to treat each parkinsonian patient, a genetically identical source of tissue may be safer and result in more predictable transplants that non-identical tissue.

Furthermore, animal cloning using cultured cells may permit the production of a gene targeted fetal tissue. Using gene targeting, rejection of xenografts may be prevented or reduced. Since xenografts attract lymphocytic infiltration, introduction of genes encoding peptides with immunosuppressant properties into the cloned tissue should reduce the chance of rejection. Introduction of genes encoding human growth factors that are neurotrophic to dopamine neurons could further improve survival of the transplants and enhance behavioral recovery.

For example, glial-cell-line-derived neurotrophic factor, basic fibroblast growth factor (bFGF), insulin-like growth factor-I, and brain-derived neurotrophic factor rescue dopamine neurons from death in tissue culture (55-59). Cotransplantation of fibroblasts transfected to produce bFGF with mesencephalic grafts greatly increases survival of the dopamine neurons in the transplants (60). Delivery of these therapeutic peptides to the brain may be possible through the transgenic expression of human growth factor genes in transplanted cloned transgenic fetal tissue.

Finally, a "suicide gene" (e.g., HSV-tk) might be introduced into cloned fetal neural tissue (61). If desired, the cell therapy could then be specifically terminated simply by initiating the suicide pathway (e.g., by administration of gancyclovir).

Thus, by simplifying the production of transgenic animals, the development and application of cloning technology for fetal tissue xenotransplantation offers many potential advantages over traditional techniques involving genetic modification of ES cell lines.

OBJECTS AND SUMMARY OF THE INVENTION

It is an object of the invention to provide novel and improved methods for xenotransplantation which utilizes organs, tissues and/or cells obtained from cloned ungulates, e.g., porcine or bovines produced by nuclear transfer using cultured differentiated bovine cells, in particular non-serum starved differentiated bovine cells as donor nuclei.

It is a more specific object of the invention to provide a novel method of xelotransplantation using organs, tissues and/or cells obtained from a cloned porcine or

bovine wherein said clone is produced by transplantation of the nucleus of a differentiated bovine cell, in particular a non-serum starved differentiated bovine or porcine cell, into an enucleated bovine or porcine oocyte.

Thus, in one aspect, the present invention provides a method for cloning a bovine or porcine (e.g., embryos, fetuses, offspring). The method comprises:

(i) inserting a desired serum or non-serum starved differentiated bovine or porcine cell or cell nucleus into an enucleated bovine oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit to yield a fused NT unit;

(ii) activating the fused NT unit to yield an activated NT unit; and

(iii) transferring said activated NT unit to a host bovine such that the NT unit develops into a fetus.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

The cells, tissues and/or organs of the resultant fetus are advantageously used in the area of cell, tissue and/or organ transplantation, or the production of desirable genotypes.

It is another object of the invention to provide a method for multiplying adult bovine having proven genetic superiority or other desirable traits.

It is another object of the invention to provide an improved method for producing genetically engineered or transgenic ungulates, e.g., porcines or bovines (i.e., NT units, fetuses, offspring). The invention also provides genetically engineered or transgenic ungulates, e.g., porcines or bovines, including those made by such a method.

It is a more specific object of the invention to provide a method for producing genetically engineered or transgenic porcine or bovine animals wherein a desired DNA sequence is inserted, removed or modified in a differentiated bovine cell or cell nucleus, which may be non-serum starved, prior to use of that differentiated cell or cell nucleus for formation of a NT unit. The invention also provides genetically engineered or transgenic bovine made by such a method.

It is another object of the invention to provide a novel method for producing ungulate CICM cells, in particular bovine or porcine CICM cells, which involves transplantation of a nucleus of a serum or non-serum

starved differentiated ungulate, e.g., porcine or bovine cells, into an enucleated cow oocyte, and then using the resulting NT unit to produce CICM cells. The invention also provides ungulate CICM cells produced by such a method.

Thus, in another aspect, the present invention provides a method for producing ungulate CICM cells. The method comprises:

(i) inserting a desired serum or non-serum starved differentiated ungulate cell or cell nucleus, e.g., a bovine or porcine cell or cell nucleus, into an enucleated ungulate oocyte, e.g., bovine or porcine oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit to yield a fused NT unit;

(ii) activating the fused NT unit to yield an activated NT unit; and

(iii) culturing cells obtained from said activated NT unit to obtain bovine CICM cells.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

It is yet another object of the invention to provide a method for producing transgenic animals having multiple

gene insertions and/or deletions by recloning. Using the above-described method, cloned ungulates, e.g., bovines or porcines, can be produced that contain one targeted deletion or insertion by effecting such deletion or insertion in a differentiated ungulate cell, e.g., a fibroblast, *in vitro*, and then utilizing the resultant transgenic differentiated cell as a nuclear donor. This method is highly efficient in the case of single gene targeting events. However, multiple gene targeting events is complicated by the fact that cells have a defined life span before they become senescent. In the case of bovine cells, the cells become senescent after about ≈ 30 population doublings.

The present invention provides a method for obviating such inefficiency by recloning. Essentially, this method will comprise subjecting a particular cell line to successive rounds of transfection, nuclear transfer, fetus production and fibroblast production.

More specifically, this will comprise producing a transgenic ungulate, e.g., a bovine or porcine by the

general methodology discussed *supra*, to produce a clone transgenic ungulate fetus;

isolating differentiated cells from the resultant cloned, transgenic ungulate fetus, e.g., fibroblasts, that comprise at least one targeted DNA deletion or insertion;

effecting a second targeted deletion or insretion *in vitro*, e.g., by electroporation of a DNA sequence into said differentiated cells that provides for a targeted insertion or deletion via homologous recombination;

using the resultant genetically manipulated cells, which comprise at least two targeted DNA deletions and/or insertions as nuclear donors; and producing a cloned transgenic fetus via nuclear transfer.

This recloning technique may be repeated as many times as required to produce transgenic ungulates containing the desired targeted deletions and/or additions. Thereby, it should be feasible to assess the effects of multiple gene additions and/or deletions, and to produce transgenic animals comprising multiple genetic modifications.

The resultant ungulate CICM cells, bovine or porcine CICM cells, are advantageously used in the area of cell,

tissue and organ transplantation, for therapy or diagnosis, and for studying development and cell differentiation. It is a specific object of the invention to use such ungulate CICM cells, e.g., bovine or porcine CICM cells, for treatment or diagnosis of any disease wherein cell, tissue or organ transplantation is therapeutically or diagnostically beneficial. The CICM cells may be used within the same species or across species.

Because CICM cells may be induced to differentiate into different cell types *in vitro*, it is another object of the invention to use cells or tissues derived from such ungulate CICM cells for treatment or diagnosis of any disease wherein cell, tissue or organ transplantation is therapeutically or diagnostically beneficial. Such diseases and injuries include Parkinson's, Huntington's, Alzheimer's, epilepsy, ALS, spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver diseases, heart disease, cartilage replacement, burns, vascular diseases, urinary tract diseases, as well as for the treatment of immune defects, bone marrow transplantation, cancer, among other diseases. The tissues may be used

within the same species or across species, for any patient in need of cell or tissue transplantation therapy.

Such a method comprises administering to or transplanting into a patient in need of such therapy at least one cell or tissue obtained or derived from a CICM line, wherein such cells may be totipotent, pluripotent or differentiated. It should be clear to those knowledgeable in the field that such a treatment may be supplemented by the administration of additional known drugs, including, but not limited to, immunosuppressants such as cyclosporin A or other any drug that increases the survival capability of the transplanted cells or tissue.

It is another specific object of the invention to use cells or tissues derived from ungulate NT units, e.g., bovine or porcine NT units, embryos, fetuses, offspring, or adult ungulates, e.g., bovines or porcines, produced according to the invention for the production of differentiated cells, tissues or organs. Such cells are also useful for the purposes described above, but are particularly useful for transplantation purposes, wherein the transplant recipient may be of the same or different species.

Although the cells and tissues from the cloned mammals are useful for treating any disease or disorder where transplantation is beneficial, in a particularly preferred embodiment, the donor cloned ungulate is a fetus, preferably a cloned bovine or porcine fetus, at least one of the transplanted cells is a fetal dopamine cell, and said cell transplantation therapy is effected to treat Parkinson's disease or a Parkinsonian-type disease. Such a method comprises:

(i) inserting a desired differentiated ungulate, e.g., bovine or porcine, cell or cell nucleus into an enucleated ungulate oocyte, e.g., bovine or porcine oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit to yield a fused NT unit;

(ii) activating said fused nuclear transfer unit to yield an activated NT unit;

(iii) transferring said activated NT unit to a host mammal such that the activated NT unit develops into a fetus;

(iv) isolating at least one dopamine cell or mesencephalic tissue from at least one fetus;

(v) transplanting said dopamine cell(s) or mesenphalic tissue into the brain of a patient with Parkinson's disease or a patient demonstrating symptoms of Parkinson's disease such that said disease symptoms are alleviated or decreased.

In particular, it is a specific object of the invention to provide a continuous, predictable source of cells and organs from cloned ungulates, in particular porcine and cattle, for transplantation purposes. Because cells derived from NT units are cloned, the cells and tissues of one cloned animal are genetically identical to those of another cloned from the same donor genetic material. Accordingly, such cells and tissues are capable of both "direct" and "indirect" self-replication and may be defined as cell lines which grow *in vivo*. Moreover, because they may be constantly regenerated using the methods according to the invention, they may be repeatedly obtained in a totipotent, pluripotent or differentiated state.

Thus, it is another specific object of the invention to provide cloned cell lines grown and maintained in an *in*

vivo environment, wherein said *in vivo* environment is a cloned ungulate, preferably a bovine or porcine. Such cell lines are distinguished from cells of a mammal that is not a clone because they have the identical genotype as another prior-existing embryonic, fetal or adult mammal that was not the product of nuclear transfer techniques. Moreover, they provide advantages over cell lines which have been adapted for long term *in vitro* growth, because such adaptation often results in genetic transformation of the cells and renders such cells unsuitable for therapeutic purposes due to acquired neoplastic or cancerous properties.

The *in vivo*-grown cell lines of the invention may be obtained from a cloned mammal at any stage of development, i.e., when the mammal is an embryo, blastocyst, fetus, new born or adult. A preferred embodiment is a differentiated cell line propagated in and isolated from cloned fetuses, wherein said cell line is a line of dopamine neuron cells. Such a cell line is obtained by a method comprising:

(i) inserting an ungulate cell or cell nucleus into an enucleated animal oocyte under conditions suitable for the formation of a nuclear transfer (NT) unit;

(ii) activating the nuclear transfer unit;

(iii) culturing said activated nuclear transfer unit past the embryonic stage until blastocysts are formed;

(iv) transferring blastocysts into a recipient female animal to allow development of a fetus; and

(v) isolating differentiated fetal dopamine neuronal cells from said fetus.

It is another specific object of the invention to use cells or tissues derived from ungulate, e.g., bovine or porcine NT units, fetuses or offspring, or ungulate CICM cells, e.g., bovine or porcine CICM cells, produced according to the invention *in vitro*, e.g. for study of cell differentiation and for assay purposes, e.g. for drug studies.

It is another object of the invention to use cells, tissues or organs produced from such tissues derived from bovine NT units, fetuses or offspring, or to provide improved methods of transplantation therapy. Such therapies include by way of example treatment of diseases

and injuries including Parkinson's, Huntington's, epilepsy, Alzheimer's, ALS, spinal cord injuries, multiple sclerosis, muscular dystrophy, diabetes, liver diseases, heart disease, cartilage replacement, burns, vascular diseases, urinary tract diseases, as well as for the treatment of immune defects, bone marrow transplantation, cancer, among other diseases.

In particular, it is a preferred embodiment of the invention to use the above-described fetal dopamine cell line grown *in vivo*, as a continuous and genetically identical source of tissue for transplantation purposes, in a method comprising administering cells of said cell line to a patient with Parkinson's disease or a Parkinsonian-type disease. Again, it should be clear to those knowledgeable in the field that such a treatment may be supplemented by the administration of additional known drugs, including, but not limited to, immunosuppressants such as cyclosporin A or other any drug that increases the survival capability of the transplanted cells or tissue.

It is another object of the invention to provide genetically engineered or transgenic tissues derived from ungulate, e.g., bovine or porcine NT units, fetuses or

offspring, or ungulate CICM cells, e.g., bovine or porcine CICM cells, produced by inserting, removing or modifying a desired DNA sequence in a differentiated bovine cell or cell nucleus prior to use of that differentiated cell or cell nucleus for formation of a NT unit.

It is another object of the invention to use the transgenic or genetically engineered tissues derived from ungulate, e.g., bovine or porcine NT units, fetuses or offspring, or ungulate, e.g., bovine or porcine CICM cells, produced according to the invention for cell therapy, in particular for the treatment and/or prevention of the diseases and injuries identified, *supra*. It is a particularly preferred embodiment to use genetically engineered fetal dopamine cells grown *in vivo* for the treatment and/or prevention of Parkinson's disease.

It should be clear to those knowledgeable in the field that such a genetic modification may be either insertion of heterologous DNA or deletion of native DNA, or any modification of the genome which increases survival of the cells or decreases or inhibits adverse immune reactions or rejection of the cells in a transplant recipient.

For instance, exemplary heterologous DNAs which would enhance transplant survival may comprise a gene encoding a growth factor, hormone, cytokine or other regulatory protein or peptide which interferes with immune recognition of the transplanted cells. Specific examples include human growth factors such as glial-cell line-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor (bFGF), insulin-like growth factor-I, and brain-derived neurotrophic factor.

A heterologous DNA according to the invention could also comprise a "suicide gene" which allows termination of therapy through targeted killing of the transplanted tissue or cell. A specific example is HSV-TK, which encodes a thymidine kinase which results in death of cells which express this protein upon administration of gancocyclovir. Other systems are known in the art; e.g., cytosine deaminase toxin, and are also encompassed in the invention.

Alternatively, the cell line may comprise a deletion ("knock-out") that prevents or inhibits expression of genes involved in rejection, e.g., MHCI, MHCII antigen genes, FAS, α 1,3 galactosyltransferase, or other genes that encode proteins that stimulate the rejection process.

Preferably, such deletions and/or insertions will be effected at target sites, e.g., by homologous recombination. Methods for introducing or deleting DNA sequences at targeted sites are known in the art.

It is another object of the invention to use the tissues derived from ungulate, e.g., bovine or porcine NT units, fetuses or offspring, or ungulate, e.g., bovine or porcine CICM cells produced according to the invention, or transgenic or genetically engineered tissues derived from ungulate NT units, fetuses or offspring, or ungulate CICM cells produced according to the invention as nuclear donors for nuclear transplantation.

It is another object of the invention to use transgenic or genetically engineered ungulate offspring, e.g., bovines or porcines, produced according to the invention in order to produce pharmacologically important proteins.

The present invention also includes a method of cloning a genetically engineered or transgenic ungulate, e.g., bovine or porcine, by which a desired DNA sequence is inserted, removed or modified in the differentiated ungulate cell or cell nucleus prior to insertion of the differentiated cow cell or cell nucleus into the enucleated

oocyte. Genetically engineered or transgenic cattle or porcines produced by such a method are advantageously used in the area of cell, tissue and/or organ transplantation, production of desirable genotypes, and production of pharmaceutical proteins. As discussed above, this procedure may be repeated as desired to introduce multiple deletions and/or insertions, preferably at targeted loci, by recloning.

Also provided by the present invention are cloned transgenic ungulates, e.g., cattle or porcine, obtained according to the above method, and offspring of those cloned, transgenic ungulates.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Sagital section through a cloned transgenic bovine fetus reveals normal fetal anatomy (A). Scale bar,

5 mm. (B) Expression of β-galactosidase detected using X-gal in fibroblasts recovered from a transgenic cloned fetus. Scale bar, 10μm. (C) PCR for the *lacZ* gene from cultured transgenic cloned mesencephalon and from transplants. Lanes: 1, 2, 3, 4, 5.

Fig. 2. Survival of TH^+ cells and β-galactosidase expression *in vitro*. Cloned and wild type bovine mesencephalons were cultured for 12 days in F12 medium with 5% human placental serum. (A) Immunocytochemistry for TH (black) and β-galactosidase (brown) revealed presence of both markers on day 5 in cultures from cloned mesencephalon. Scale bar, 20 μm. (B) In cultures from wild type mesencephalon TH^+ cells survived in culture, but their numbers decreased over the two week course of the experiment. The half-life was 5.6 days for wild type TH^+ cells and 4.1 days for the cloned TH^+ cells.

Fig. 3. Rotational behavior and TH^+ cell survival following transplantation of transgenic cloned mesencephalon and vehicle in parkinsonian rats (A). Animals were injected with 5.0 mg/kg methamphetamine prior to the transplant (100% rotation), one month, and two

months after transplant. Transplants of cloned mesencephalon significantly reduced the rotational behavior in the parkinsonian rats. (B) Relationship between the behavioral improvement and TH^+ cell survival in the grafts from both cloned and wild type mesencephalon. (C) Comparison of maximum fiber span (mm) in wild-type, cloned and host striatum.

Fig. 4. Combined TH immunocytochemistry and hematoxylin and eosin (H&E) staining of cloned transgenic mesencephalic graft. (A) overall modest inflammation is distributed by rosette-like groups of infiltrating lymphocytes. (B) Some cells appear to contain spheres of condensed chromatin indicative of apoptotic cell death (arrow). Scale bar: (A), 200 μm; (B) 50 μm.

Fig. 5. Transplant morphology showing distribution of transplanted TH^+ cells. (A, B) TH immunocytochemistry of a cloned mesencephalic transplant. A significant number of neurites extend from the graft into the recipient's striatum. (C, D) TH immunocytochemistry of a wild type mesencephalic transplant. (E) TH immunocytochemistry of a vehicle transplant. Scale bar: (A, C, and E) 2.0 mm; (B and D) 0.5 mm.

Fig. 6. Schematic of recloning approach used to engineer multiple gene targeting events.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides improved cloning procedures in which cell nuclei derived from differentiated fetal or adult ungulate cells, e.g., bovine or porcine, which may be serum or non-serum starved are transplanted into enucleated oocytes of the same species as the donor nuclei. However, prior to discussing this invention in further detail, the following terms will first be defined.

Definitions

As used herein, the following terms have the following meanings:

The term "differentiated" refers to cells having a different character or function from the surrounding structures or from the cell of origin. Differentiated ungulate cells are those cells which are past the early embryonic stage. More particularly, the differentiated cells are those from at least past the embryonic disc stage (day 10 of bovine embryogenesis). The differentiated cells may be derived from ectoderm, mesoderm or endoderm.

The term "nuclear transfer" or "nuclear transplantation" refers to a method of cloning wherein the nucleus from a donor cell is transplanted into enucleated oocytes. Nuclear transfer techniques or nuclear transplantation techniques are known in the literature.[3,7,16,27,35-37] Also, U.S. Patent Nos. 4,994,384 and 5,057,420 describe procedures for bovine nuclear transplantation. In the subject application, nuclear transfer or nuclear transplantation or NT are used interchangeably.

The term "cloned" in reference to the cells, tissues and animals of the invention means that such cells, tissues and animals were obtained by nuclear transplantation techniques.

The term "nuclear transfer unit" or "NT unit" refers to the product of fusion between a differentiated ungulate cell or cell nucleus, e.g., bovine or porcine cell or cell nucleus, and an enucleated ungulate oocyte, e.g., bovine or porcine oocyte, and is sometimes referred to herein as a fused NT unit.

The term "non-serum starved bovine differentiated cells" refers to cells cultured in the presence of serum greater than about 1%.

The term "fetus" refers to the unborn young of a viviporous animal after it has taken form in the uterus. In cattle, the fetal stage occurs from 35 days after conception until birth.

The term "adult" refers to a mammal from birth until death.

The term "patient" refers to any mammal, including ungulates, rodents and humans, which would benefit from the therapies of the invention.

The term "Parkinsonian-type disease" refers to any disease or disorder which produces symptoms normally associated with Parkinson's disease, wherein the patient demonstrating such symptoms would benefit from transplantation therapy of fetal dopamine cells.

The term "*in vivo* environment" as it applies to growing and maintaining the cell lines of the invention refers to the body of a mammal, preferably a bovine or porcine. Such a mammal may be an embryo, fetus, new born or adult. When using "*in vivo* environment to refer to an embryo or fetus, the term generally refers to the cloned embryo or fetus and not the recipient or host female.

The terms "direct and indirect self-replication" when referring to the cell lines, tissues and mammals of the invention is in accordance with the definition of biological material set forth in 37 CFR §1.801.

According to the invention, cell nuclei derived from differentiated ungulate cells, e.g., bovine or porcine, are transplanted into enucleated cow oocytes. The nuclei are reprogrammed to direct the development of cloned embryos, which can then be transferred into recipient females to produce fetuses and offspring, or used to produce CICM cells. The cloned embryos can also be combined with fertilized embryos to produce chimeric embryos, fetuses and/or offspring.

Prior art methods have used embryonic cell types in cloning procedures. This includes work by Campbell, *et al.*[4] and Stice, *et al.*[31] In both of those studies, embryonic cell lines were derived from embryos of less than 10 days of gestation. In both studies, the cells were maintained on a feeder layer to prevent overt differentiation of the donor cell to be used in the cloning procedure. The present invention uses differentiated cells.

Adult cells and fetal fibroblast cells from a sheep have purportedly been used to produce sheep offspring.[34] However, of the mammalian species studied, cloning of sheep appears to be the easiest, and pig cloning appears to be the most difficult. The successful cloning of cows using differentiated cell types according to the present invention was quite unexpected.

Thus, according to the present invention, multiplication of superior genotypes of ungulates, e.g., porcines and bovines, is possible. This will allow the multiplication of adult ungulates with proven genetic superiority or other desirable traits. Genetic progress will be accelerated in the cow. By the present invention, there are potentially billions of fetal or adult ungulate cells, e.g., porcine or bovine cells, that can be harvested and used in the cloning procedure. This will potentially result in many identical offspring in a short period.

It was unexpected that cloned embryos with fetal or adult donor nuclei could develop to advanced embryonic and fetal stages. The scientific dogma has been that only early embryonic cell types could direct this type of development. It was further unexpected that a large number of

cloned embryos could be produced from fetal or adult cells. Still further, the fact that new transgenic embryonic cell lines could be readily derived from transgenic cloned embryos was unexpected.

Adult cells and fetal fibroblast cells from a sheep have purportedly been used to produce a sheep offspring (Wilmut et al, 1997). In that study, however, it was emphasized that the use of a serum starved, nucleus donor cell in the quiescent state was important for success of the Wilmut cloning method. No such requirement for serum starvation or quiescence exists for the present invention. To the contrary, cloning is achieved using non-serum starved, differentiated mammalian cells. Moreover, cloning efficiency according to the present invention can be the same regardless of whether fetal or adult donor cells are used, whereas Wilmut et al (1997) reported that lower cloning efficiency was achieved with adult donor cells.

There has also been speculation that the Wilmut, *et al.* method will lead to the generation of transgenic animals.[17] However, there is no reason to assume, for example, that nuclei from adult cells that have been transfected

with exogenous DNA will be able to survive the process of nuclear transfer. In this regard, it is known that the properties of mouse embryonic stem (ES) cells are altered by *in vitro* manipulation such that their ability to form viable chimeric embryos is effected. Therefore, prior to the present invention, the cloning of transgenic animals could not have been predicted.

The present invention also allows simplification of transgenic procedures by working with a cell source that can be clonally propagated. This eliminates the need to maintain the cells in an undifferentiated state, thus, genetic modifications, both random integration and gene targeting, are more easily accomplished. Also by combining nuclear transfer with the ability to modify and select for these cells *in vitro*, this procedure is more efficient than previous transgenic embryo techniques. According to the present invention, these cells can be clonally propagated without cytokines, conditioned media and/or feeder layers, further simplifying and facilitating the transgenic procedure. When transfected cells are used in cloning procedures according to the invention, transgenic NT embryos are

produced which can develop into fetuses and offspring. Also, these transgenic cloned embryos can be used to produce CICM cell lines or other embryonic cell lines. Therefore, the present invention eliminates the need to derive and maintain *in vitro* an undifferentiated cell line that is conducive to genetic engineering techniques.

The present invention can also be used to produce cloned ungulate fetuses, offspring or CICM cells which can be used, for example, in cell, tissue and organ transplantation. By taking a fetal or adult cell from an ungulate, e.g., porcine or bovine, and using it in the cloning procedure a variety of cells, tissues and possibly organs can be obtained from cloned fetuses as they develop through organogenesis. Cells, tissues, and organs can be isolated from cloned offspring as well. This process can provide a source of "materials" for many medical and veterinary therapies including cell and gene therapy. If the cells are transferred back into the animal in which the cells were derived, then immunological rejection is averted. Also, because many cell types can be isolated from these clones, other methodologies such as hematopoietic chimerism can be used to avoid immunological rejection

among animals of the same species as well as between species.

Thus, in one aspect, the present invention provides a method for cloning an ungulate, e.g., a bovine or porcine. In general, the cloned ungulate, e.g., porcine or bovine, will be produced by a nuclear transfer process comprising the following steps:

(i) obtaining desired differentiated cow cells, which may be serum or non-serum starved, to be used as a source of donor nuclei;

(ii) obtaining oocytes from an ungulate, e.g., bovine or porcine;

(iii) enucleating said oocytes;

(iv) transferring the desired differentiated cell or cell nucleus into the enucleated oocyte, e.g., by fusion or injection, to form an NT unit;

(v) activating the NT unit to yield an activated NT unit; and

(vi) transferring said activated NT unit to a host ungulate, e.g., porcine or bovine, such that the NT unit develops into a fetus.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage prior to transfer to the host ungulate.

The present invention also includes a method of cloning a genetically engineered or transgenic ungulate, e.g., porcine or bovine, by which a desired DNA sequence is inserted, removed or modified in the serum or non-serum starved differentiated ungulate cell or cell nucleus prior to insertion of the differentiated ungulate cell or cell nucleus into the enucleated ungulate oocyte.

Also provided by the present invention are transgenic ungulates obtained according to the above method, and offspring of those cloned, transgenic ungulates.

In addition to the uses described above, the genetically engineered or transgenic ungulates according to the invention can be used to produce a desired protein, such as a pharmacologically important protein, e.g., human serum albumin. That desired protein can then be isolated from the milk or other fluids or tissues of the transgenic ungulate, preferably a bovine. Alternatively, the exogenous DNA sequence may confer an agriculturally useful trait to the transgenic ungulate, e.g., bovine or porcine,

such as disease resistance, decreased body fat, increased lean meat product, improved feed conversion, or altered sex ratios in progeny.

In another aspect, the present invention provides a method for producing ungulate CICM cells. The method comprises:

(i) inserting a desired serum or non-serum starved differentiated ungulate, e.g., bovine or porcine, cell or cell nucleus into an enucleated ungulate oocyte, under conditions suitable for the formation of a nuclear transfer (NT) unit;

(ii) activating the resultant nuclear transfer unit to yield an activated nuclear transfer unit; and

(iii) culturing cells obtained from said activated NT unit to obtain ungulate, e.g., porcine or bovine, CICM cells.

Optionally, the activated nuclear transfer unit is cultured until greater than the 2-cell developmental stage.

The resultant ungulate CICM cells are advantageously used in the area of cell, tissue and organ transplantation, or in the production of fetuses or offspring, including transgenic fetuses or offspring.

Preferably, the NT units will be cultured to a size of at least 2 to 400 cells, preferably 4 to 128 cells, and most preferably to a size of at least about 50 cells.

The present invention further provides for the use of NT fetuses and NT ungulate animals and chimeric offspring in the area of cell, tissue and organ transplantation, and envision the cells, tissues organs of NT mammals as a continuous and reproducible source of therapeutic products. Accordingly, such cells and tissues are specifically described as maintainable cell lines grown *in vivo*.

A preferred embodiment is a fetal dopamine cell line maintained *in vivo*, which may be used for transplantation into and treatment of patients with Parkinson's disease or Parkinsonian-type diseases. In particular, xenotransplantation into a human patient is envisioned.

Ungulate cells to serve as nuclear donors may be obtained by well known methods. Ungulate, e.g., bovine or porcine, cells useful in the present invention include, by way of example, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes),

erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. Moreover, the ungulate cells used for nuclear transfer may be obtained from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc. These are just examples of suitable donor cells. Suitable donor cells, i.e., cells useful in the subject invention, may be obtained from any cell or organ of the body. This includes all somatic or germ cells.

Fibroblast cells are an ideal cell type because they can be obtained from developing fetuses and adult ungulates, e.g., porcines and bovines, in large quantities. Fibroblast cells are differentiated somewhat and, thus, were previously considered a poor cell type to use in cloning procedures. Importantly, these cells can be easily propagated *in vitro* with a rapid doubling time and can be clonally propagated for use in gene targeting procedures. Again the present invention is novel because differentiated cell types are used. The present invention is advantageous

because the cells can be easily propagated, genetically modified and selected *in vitro*.

Other reported cloning methods (e.g., Wilmut et al, 1997) have relied on the use of serum starved cells. The present invention, however, includes the use of donor cells which are not in a state of serum starvation. According to Wilmut et al (1997), serum starved cells are quiescent, i.e., exiting the growth phase. Other methods (chemical, temperature, etc.) are also capable of producing quiescent cells. By contrast, in the present invention the donor cells used may or may not be quiescent.

The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be significant to the success of NT methods.[23] In general, successful mammalian embryo cloning practices use the metaphase II stage oocyte as the recipient oocyte because at this stage it is believed that the oocyte can be or is sufficiently "activated" to treat the introduced nucleus as it does a fertilizing sperm. In domestic animals, the oocyte activation period generally ranges from about 16-52 hours, preferably about 20-45 hours post-aspiration.

Methods for isolation of oocytes are well known in the art. Essentially, this will comprise isolating oocytes from the ovaries or reproductive tract of an ungulate, e.g., a bovine. A readily available source of bovine oocytes is slaughterhouse materials.

For the successful use of techniques such as genetic engineering, nuclear transfer and cloning, oocytes are preferably matured *in vitro* before these cells are used as recipient cells for nuclear transfer, and before they can be fertilized by the sperm cell to develop into an embryo. In the case of bovines, this process generally requires collecting immature (prophase I) oocytes from mammalian ovaries, e.g., bovine ovaries obtained at a slaughterhouse, and maturing the oocytes in a maturation medium prior to fertilization or enucleation until the oocyte attains the metaphase II stage, which in the case of bovine oocytes generally occurs about 18-24 hours post-aspiration. For purposes of the present invention, this period of time is known as the "maturation period." As used herein for calculation of time periods, "aspiration" refers to aspiration of the immature oocyte from ovarian follicles.

Alternatively, metaphase II stage oocytes, which have been matured *in vivo* can be successfully used in the subject nuclear transfer techniques. Essentially, mature metaphase II oocytes are collected surgically from either non-superovulated or superovulated ungulates, e.g., cows or heifers 35 to 48 hours past the onset of estrus or past the injection of human chorionic gonadotropin (hCG) or similar hormone.

While the subject techniques should be generically suitable for cloning any ungulate, the following discussion focuses on the production of cloned bovines. As discussed above, the methodology for producing cloned porcines, which is highly similar, is disclosed in U.S. Serial No. 08/888,057, which is incorporated by reference in its entirety herein.

The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be significant to the success of NT methods. (*See* e.g., Prather et al., *Differentiation*, 48, 1-8, 1991). In general, successful mammalian embryo cloning practices use the metaphase II stage oocyte as the recipient oocyte because at this stage

it is believed that the oocyte can be or is sufficiently "activated" to treat the introduced nucleus as it does a fertilizing sperm. In domestic animals, and especially cattle, the oocyte activation period generally ranges from about 16-52 hours, preferably about 28-42 hours post-aspiration.

For example, immature oocytes may be washed in HEPES buffered hamster embryo culture medium (HECM) as described in Seshagine et al., *Biol. Reprod.*, 40, 544-606, 1989, and then placed into drops of maturation medium consisting of 50 microliters of tissue culture medium (TCM) 199 containing 10% fetal calf serum which contains appropriate gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and estradiol under a layer of lightweight paraffin or silicon at 39°C.

After a fixed time maturation period, which ranges from about 10 to 40 hours, and preferably about 16-18 hours, the oocytes will be enucleated. Prior to enucleation the oocytes will preferably be removed and placed in HECM containing 1 milligram per milliliter of hyaluronidase prior to removal of cumulus cells. This may be effected by repeated pipetting through very fine bore pipettes or by

vortexing briefly. The stripped oocytes are then screened for polar bodies, and the selected metaphase II oocytes, as determined by the presence of polar bodies, are then used for nuclear transfer. Enucleation follows.

Enucleation may be effected by known methods, such as described in U.S. Patent No. 4,994,384 which is incorporated by reference herein. For example, metaphase II oocytes are either placed in HECM, optionally containing 7.5 micrograms per milliliter cytochalasin B, for immediate enucleation, or may be placed in a suitable medium, for example an embryo culture medium such as CR1aa, plus 10% estrus cow serum, and then enucleated later, preferably not more than 24 hours later, and more preferably 16-18 hours later.

Enucleation may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm. The oocytes may then be screened to identify those of which have been successfully enucleated. This screening may be effected by staining the oocytes with 1 microgram per milliliter 33342 Hoechst dye in HECM, and then viewing the oocytes under ultraviolet irradiation for less than 10 seconds. The oocytes that have been success-

fully enucleated can then be placed in a suitable culture medium, e.g., CR1aa plus 10% serum.

In the present invention, the recipient oocytes will preferably be enucleated at a time ranging from about 10 hours to about 40 hours after the initiation of *in vitro* maturation, more preferably from about 16 hours to about 24 hours after initiation of *in vitro* maturation, and most preferably about 16-18 hours after initiation of *in vitro* maturation.

A single mammalian cell of the same species as the enucleated oocyte will then be transferred into the peri-vitelline space of the enucleated oocyte used to produce the NT unit. The mammalian cell and the enucleated oocyte will be used to produce NT units according to methods known in the art. For example, the cells may be fused by electrofusion. Electrofusion is accomplished by providing a pulse of electricity that is sufficient to cause a transient breakdown of the plasma membrane. This breakdown of the plasma membrane is very short because the membrane reforms rapidly. Thus, if two adjacent membranes are induced to breakdown and upon reformation the lipid

bilayers intermingle, small channels will open between the two cells. Due to the thermodynamic instability of such a small opening, it enlarges until the two cells become one. Reference is made to U.S. Patent 4,997,384 by Prather et al. (incorporated by reference in its entirety herein), for a further discussion of this process. A variety of electrofusion media can be used including e.g., sucrose, mannitol, sorbitol and phosphate buffered solution. Fusion can also be accomplished using Sendai virus as a fusogenic agent (Graham, *Wister Inot. Symp. Monogr.*, 9, 19, 1969).

Also, in some cases (e.g. with small donor nuclei) it may be preferable to inject the nucleus directly into the oocyte rather than using electroporation fusion. Such techniques are disclosed in Collas and Barnes, *Mol. Reprod. Dev.*, 38:264-267 (1994), incorporated by reference in its entirety herein.

Preferably, the bovine cell and oocyte are electrofused in a 500 μm chamber by application of an electrical pulse of 90-120V for about 15 μsec, about 24 hours after initiation of oocyte maturation. After fusion, the resultant fused NT units are then placed in a suitable medium

until activation, e.g., CR1aa medium. Typically activation will be effected shortly thereafter, preferably less than 24 hours later, and more preferably about 4-9 hours later.

The NT unit may be activated by known methods. Such methods include, e.g., culturing the NT unit at sub-physiological temperature, in essence by applying a cold, or actually cool temperature shock to the NT unit. This may be most conveniently done by culturing the NT unit at room temperature, which is cold relative to the physiological temperature conditions to which embryos are normally exposed.

Alternatively, activation may be achieved by application of known activation agents. For example, penetration of oocytes by sperm during fertilization has been shown to activate prefusion oocytes to yield greater numbers of viable pregnancies and multiple genetically identical calves after nuclear transfer. Also, treatments such as electrical and chemical shock may be used to activate NT embryos after fusion. Suitable oocyte activation methods are the subject of U.S. Patent No. 5,496,720, to Susko-Parrish et al., herein incorporated by reference in its entirety.

Additionally, activation may be effected by simultaneously or sequentially conducting the following steps, in either order:

(i) increasing levels of divalent cations in the oocyte, and

(ii) reducing phosphorylation of cellular proteins in the oocyte.

This will generally be effected by introducing divalent cations into the oocyte cytoplasm, e.g., magnesium, strontium, barium or calcium, e.g., in the form of an ionophore. Other methods of increasing divalent cation levels include the use of electric shock, treatment with ethanol and treatment with caged chelators.

Phosphorylation may be reduced by known methods, e.g., by the addition of kinase inhibitors, e.g., serine-threonine kinase inhibitors, such as 6-dimethylaminopurine, staurosporine, 2-aminopurine, and sphingosine.

Alternatively, phosphorylation of cellular proteins may be inhibited by introduction of a phosphatase into the oocyte, e.g., phosphatase 2A and phosphatase 2B.

In one embodiment, NT activation is effected by briefly exposing the fused NT unit to a TL-HEPES medium contain-

ing 5μM ionomycin and 1 mg/ml BSA, followed by washing in TL-HEPES containing 30 mg/ml BSA within about 24 hours after fusion, and preferably about 4 to 9 hours after fusion.

The activated NT units may then be cultured in a suitable *in vitro* culture medium until the generation of CICM cells and cell colonies. Culture media suitable for culturing and maturation of embryos are well known in the art. Examples of known media, which may be used for bovine embryo culture and maintenance, include Ham's F-10 + 10% fetal calf serum (FCS), Tissue Culture Medium-199 (TCM-199) + 10% fetal calf serum, Tyrodes-Albumin-Lactate-Pyruvate (TALP), Dulbecco's Phosphate Buffered Saline (PBS), Eagle's and Whitten's media. One of the most common media used for the collection and maturation of oocytes is TCM-199, and 1 to 20% serum supplement including fetal calf serum, newborn serum, estrual cow serum, lamb serum or steer serum. A preferred maintenance medium includes TCM-199 with Earl salts, 10% fetal calf serum, 0.2 mM Na pyruvate and 50 μg/ml gentamicin sulphate. Any of the above may also involve co-culture with a variety of cell types such as

granulosa cells, oviduct cells, BRL cells and uterine cells and STO cells.

Another maintenance medium is described in U.S. Patent 5,096,822 to Rosenkrans, Jr. et al., which is incorporated herein by reference. This embryo medium, named CR1, contains the nutritional substances necessary to support an embryo.

CR1 contains hemicalcium L-lactate in amounts ranging from 1.0 mM to 10 mM, preferably 1.0 mM to 5.0 mM. Hemicalcium L-lactate is L-lactate with a hemicalcium salt incorporated thereon. Hemicalcium L-lactate is significant in that a single component satisfies two major requirements in the culture medium: (i) the calcium requirement necessary for compaction and cytoskeleton arrangement; and (ii) the lactate requirement necessary for metabolism and electron transport. Hemicalcium L-lactate also serves as valuable mineral and energy source for the medium necessary for viability of the embryos.

Advantageously, CR1 medium does not contain serum, such as fetal calf serum, and does not require the use of a co-culture of animal cells or other biological media, i.e., media comprising animal cells such as oviductal cells.

Biological media can sometimes be disadvantageous in that they may contain microorganisms or trace factors which may be harmful to the embryos and which are difficult to detect, characterize and eliminate.

Examples of the main components in CR1 medium include hemicalcium L-lactate, sodium chloride, potassium chloride, sodium bicarbonate and a minor amount of fatty-acid free bovine serum albumin (Sigma A-6003). Additionally, a defined quantity of essential and non-essential amino acids may be added to the medium. CR1 with amino acids is known by the abbreviation "CR1aa".

CR1 medium preferably contains the following components in the following quantities:

sodium chloride	- 114.7 mM
potassium chloride	- 3.1 mM
sodium bicarbonate	- 26.2 mM
hemicalcium L-lactate	- 5 mM
fatty-acid free BSA	- 3 mg/ml

In one embodiment, the activated NT embryos unit are placed in CR1aa medium containing 1.9 mM DMAP for about 4 hours followed by a wash in HECM and then cultured in CR1aa containing BSA.

For example, the activated NT units may be transferred to CR1aa culture medium containing 2.0 mM DMAP (Sigma) and cultured under ambient conditions, e.g., about 38.5°C, 5% CO_2 for a suitable time, e.g., about 4 to 5 hours.

Afterward, the cultured NT unit or units are preferably washed and then placed in a suitable media, e.g., CR1aa medium containing 10% FCS and 6 mg/ml contained in well plates which preferably contain a suitable confluent feeder layer. Suitable feeder layers include, by way of example, fibroblasts and epithelial cells, e.g., fibroblasts and uterine epithelial cells derived from ungulates, chicken fibroblasts, murine (e.g., mouse or rat) fibroblasts, STO and SI-m220 feeder cell lines, and BRL cells.

In one embodiment, the feeder cells comprise mouse embryonic fibroblasts. Preparation of a suitable fibroblast feeder layer is described in the example which follows and is well within the skill of the ordinary artisan.

The methods for embryo transfer and recipient animal management in the present invention are standard procedures used in the embryo transfer industry. Synchronous transfers are important for success of the present invention, i.e., the stage of the NT embryo is in synchrony with the

estrus cycle of the recipient female. This advantage and how to maintain recipients are reviewed in Siedel, G.E., Jr. ("Critical review of embryo transfer procedures with cattle" in Fertilization and Embryonic Development in Vitro (1981) L. Mastroianni, Jr. and J.D. Biggers, ed., Plenum Press, New York, NY, page 323), the contents of which are hereby incorporated by reference.

The present invention can also be used to clone genetically engineered or transgenic ungulates, in particular cattle and porcines. As explained above, the present invention is advantageous in that transgenic procedures can be simplified by working with a differentiated cell source that can be clonally propagated. In particular, the differentiated cells used for donor nuclei, which may or may not be serum-starved, have a desired DNA sequence inserted, removed or modified. Those genetically altered, differentiated cells are then used for nuclear transplantation with enucleated oocytes. Moreover, as discussed above, this cloning procedure can be repeated to introduce multiple gene deletions or additions.

Any known method for inserting, deleting or modifying a desired DNA sequence from a mammalian cell may be used

for altering the differentiated cell to be used as the nuclear donor. These procedures may remove all or part of a DNA sequence, and the DNA sequence may be heterologous. Included is the technique of homologous recombination, which allows the insertion, deletion or modification of a DNA sequence or sequences at a specific site or sites in the cell genome.

The present invention can thus be used to provide adult ungulates, e.g., bovines or porcines, with desired genotypes. Multiplication of adult ungulates, e.g., bovines or porcines, with proven genetic superiority or other desirable traits is particularly useful, including transgenic or genetically engineered animals, and chimeric animals. Thus, the present invention will allow production of single sex offspring, and production of ungulates having improved meat production, reproductive traits and disease resistance. Furthermore, cell and tissues from the NT fetus, including transgenic and/or chimeric fetuses, can be used in cell, tissue and organ transplantation for the treatment of numerous diseases as described below. Hence, transgenic ungulates, in particular porcines or bovines, have uses including models for diseases, xenotrans-

plantation of cells and organs, and production of pharmaceutical proteins.

For production of CICM cells and cell lines, the activated NT units are cultured under conditions which promote cell division without differentiation to provide for cultured NT units. After cultured NT units of the desired size are obtained, the cells are mechanically removed from the zone and are then used. This is preferably effected by taking the clump of cells which comprise the cultured NT unit, which typically will contain at least about 50 cells, washing such cells, and plating the cells onto a feeder layer, e.g., irradiated fibroblast cells. Typically, the cells used to obtain the stem cells or cell colonies will be obtained from the inner most portion of the cultured NT unit which is preferably at least 50 cells in size. However, cultured NT units of smaller or greater cell numbers as well as cells from other portions of the cultured NT unit may also be used to obtain ES cells and cell colonies. The cells are maintained on the feeder layer in a suitable growth medium, e.g., alpha MEM supplemented with 10% FCS and 0.1 mM ß-mercaptoethanol (Sigma)

and L-glutamine. The growth medium is changed as often as necessary to optimize growth, e.g., about every 2-3 days.

This culturing process results in the formation of CICM cells or cell lines. One skilled in the art can vary the culturing conditions as desired to optimize growth of the particular CICM cells. Also, genetically engineered or transgenic ungulate CICM cells may be produced according to the present invention. That is, the methods described above can be used to produce NT units in which a desired DNA sequence or sequences have been introduced, or from which all or part of an endogenous DNA sequence or sequences have been removed or modified. Those genetically engineered or transgenic NT units can then be used to produce genetically engineered or transgenic CICM cells.

The resultant CICM cells and cell lines have numerous therapeutic and diagnostic applications. Most especially, such CICM cells may be used for cell transplantation therapies.

In this regard, it is known that mouse embryonic stem (ES) cells are capable of differentiating into almost any cell type, e.g., hematopoietic stem cells. Therefore, cow CICM cells produced according to the invention should

possess similar differentiation capacity. The CICM cells according to the invention will be induced to differentiate to obtain the desired cell types according to known methods. For example, the subject ungulate CICM cells may be induced to differentiate into hematopoietic stem cells, neural cells, muscle cells, cardiac muscle cells, liver cells, cartilage cells, epithelial cells, urinary tract cells, neural cells, etc., by culturing such cells in differentiation medium and under conditions which provide for cell differentiation. Medium and methods which result in the differentiation of CICM cells are known in the art as are suitable culturing conditions.

For example, Palacios, *et al.*[21] teaches the production of hematopoietic stem cells from an embryonic cell line by subjecting stem cells to an induction procedure comprising initially culturing aggregates of such cells in a suspension culture medium lacking retinoic acid followed by culturing in the same medium containing retinoic acid, followed by transferral of cell aggregates to a substrate which provides for cell attachment.

Moreover, Pedersen[22] is a review article which references numerous articles disclosing methods for *in vitro*

differentiation of embryonic stem cells to produce various differentiated cell types including hematopoietic cells, muscle, cardiac muscle, nerve cells, among others.

Further, Bain, *et al.*[1] teaches *in vitro* differentiation of embryonic stem cells to produce neural cells which possess neuronal properties. These references are exemplary of reported methods for obtaining differentiated cells from embryonic or stem cells. These references and in particular the disclosures therein relating to methods for differentiating embryonic stem cells are incorporated by reference in their entirety herein.

Thus, using known methods and culture mediums, one skilled in the art may culture the subject CICM cells, including genetically engineered or transgenic CICM cells, to obtain desired differentiated cell types, e.g., neural cells, muscle cells, hematopoietic cells, etc.

The subject CICM cells may be used to obtain any desired differentiated cell type. Therapeutic usages of such differentiated cells are unparalleled. For example, hematopoietic stem cells may be used in medical treatments requiring bone marrow transplantation. Such procedures are used to treat many diseases, e.g., late stage cancers such

as ovarian cancer and leukemia, as well as diseases that compromise the immune system, such as AIDS. Hematopoietic stem cells can be obtained, e.g., by fusing adult somatic cells of a cancer or AIDS patient, e.g., epithelial cells or lymphocytes with an enucleated oocyte, obtaining CICM cells as described above, and culturing such cells under conditions which favor differentiation, until hematopoietic stem cells are obtained. Such hematopoietic cells may be used in the treatment of diseases including cancer and AIDS.

The cells of the present invention can be used to replace defective genes, e.g., defective immune system genes, or to introduce genes which result in the expression of therapeutically beneficial proteins such as growth factors, lymphokines, cytokines, enzymes, etc.

DNA sequences which may be introduced into the subject CICM cells include, by way of example, those which encode epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, insulin-like growth factor (I and II), neurotrophin-3, neurotrophin-4/5, ciliary neurotrophic factor, AFT-1, cytokines (interleukins, interferons, colony stimulating factors, tumor necro-

sis factors (alpha and beta), etc.), therapeutic enzymes, etc.

The present invention includes the use of ungulate cells in the treatment of human diseases. Thus, ungulate CICM cells, NT fetuses and NT ungulates and chimeric offspring (transgenic or non-transgenic) may be used in the treatment of human disease conditions where cell, tissue or organ transplantation is warranted. In general, CICM cells, fetuses and offspring according to the present invention can be used within the same species (autologous, syngenic or allografts) or across species (xenografts). In a preferred embodiment, brain cells from porcine or bovine NT fetuses are used to treat Parkinson's disease.

Also, the subject CICM cells may be used as an *in vitro* model of differentiation, in particular for the study of genes which are involved in the regulation of early development. Also, differentiated cell tissues and organs using the subject CICM cells may be used in drug studies.

Further, the subject CICM cells may be used as nuclear donors for the production of other CICM cells and cell colonies.

The use of cells obtained from NT fetuses and offspring rather than from CICM cell lines may provide advantages in the area of xenotransplantation when medium components required for differentiation of a particular cell type are not yet known, or difficult to obtain. In addition, tissues and whole organs may be more easily obtained from cloned fetuses and adult ungulates, e.g., cattle or porcines, than from differentiated cells growing in culture. Moreover, cells, tissues and organs from cloned ungulate fetuses and adult animals are equally as useful for transplantation therapies as described for the subject CICM cells above.

In a particularly preferred embodiment, dopamine cells from transgenic cloned fetuses are used for xenotransplantation into patients with Parkinson's disease or a Parkinsonian-type disease. The present invention describes in an exemplary fashion the generation of cloned transgenic bovine embryos by fusing *lacZ*-transfected bovine fibroblasts with enucleated bovine oocytes. The embryos were transferred into surrogate cows, and a high proportion of established pregnancies developed past 40 days (38%). Dopamine cells collected from the ventral mesencephalon of

cloned transgenic bovine fetuses 42 to 50 days post conception survived transplantation into immunosuppressed parkinsonian rats. Cells from cloned and wild type embryos improved motor performance in rats. The *lacZ* gene was detected in the transplanted cloned mesencephalon. These results demonstrate that somatic cell cloning may be used to produce transgenic animal tissue for treatment of parkinsonism.

In order to more clearly describe the subject invention, the following examples are provided.

EXAMPLES

MATERIALS AND METHODS FOR BOVINE CLONING

Modified TL-Hepes-PVA Medium (Hepes-PVA)

Component	Mol. Wt.	Conc. (mM)	g/l
NaCl	58.45	114.00	6.6633
KCl	74.55	3.20	0.2386
$NaHCO_3$	84.00	2.00	0.1680
NaH_2PO_4	120.00	0.34	0.0408
Na Lactate**	112.10	10.00	1.868 ml
$MgCl_26H_2O$	203.30	0.50	0.1017
$CaCl_22H_2O$*	147.00	2.00	0.2940
Sorbitol	182.20	12.00	2.1864

Component	Mol. Wt.	Conc. (mM)	g/l
HEPES	238.30	10.00	2.3830
Na Pyruvate	110.00	0.20	0.0220
Gentamycin	----	----	500 μl
Penicillin G	----	----	0.0650
PVA	10,000	----	0.1000

**60% syrup
* Add $CaCl_2 2H_2O$ last, slowly to prevent precipitation
Use 18 mohm, RO, DI water.
Adjust pH to 7.4, Check osmolarity and record.
Sterilize by vacuum filtration (0.22 μm), date and initial bottle.
Store at 4°C and use within 10 days.

B_2 MEDIUM

B_2 Medium is a ready-to-use synthetic medium conventionally used for cell culture, processing and handling of human sperm.

Composition:

Mineral Salts: KCl, NaCl, $MgSO_4$, $NaHCO_3$, Na_2HPO_4, KH_2PO_4.

Amino Acids: Asparagine, threonine, serine, glutamic acid, glycine, alanine, taurine, citrulline, valine, cystine, methionine, isoleucine, leucine, tyrosine, arginine, phenylalanine, ornithine, lysine, tryptophan, arginine, histidine, proline, and cysteine.

Albumin: 10g/L Bovine serum albumin(BSA)

Lipid: Cholesterol

Sugars and metabolic by-products: Glucose, pyruvate, lactate, and acetate

Vitamins and ascorbic acid

Purine and pyrimidine bases

Antibiotics: 100 mg/liter of penicillin G and 40 mg/liter of streptomycin

Phenol Red: 15 milligrams/liter

pH: 7.2 - 7.5

Osmolarity: 275-305 mOsm/Kg

Antibiotic/Antimycotic (Ab/Am)

100 U/1 Penicillin, 100 μg/l streptomycin and 0.25 μg/l amphotericin B (Gibco #15240-062)

Add a 10 ml aliquot to each liter of saline.

Add 10 μl to each ml of semen.

Oocyte-Cumulus Complex (OCC) Collection

Ovaries are transported to the lab at 25°C and immediately washed with 0.9% saline with antibiotic/antimycotic (10 ml/L; Gibco #600-5240g). Follicles between 3-6 mm are aspirated using 18g needles and 50 ml Falcon tubes connected to vacuum system (GEML bovine system). After tube is filled, OCC's are allowed to settle for 5-10 minutes. Follicular fluid (bFF) is aspirated and saved for use in

culture system if needed (see bFF preparation protocol below).

OCC Washing

OCCs are resuspended in 20 ml Hepes-PVA and allowed to settle; repeat 2 times. After last wash, OCCs are moved to grid dishes and selected for culture. Selected OCCs are washed twice in 60 mm dishes of Hepes-PVA. All aspiration and oocyte recovery are performed at room temperature (approx. 25°C).

Isolation of primary cultures of bovine embryonic and adult fibroblast cells

Primary cultures of bovine fibroblasts are obtained from cow fetuses 30 to 114 days postfertilization, preferably 45 days. The head, liver, heart and alimentary tract are aseptically removed, the fetuses minced and incubated for 30 minutes at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells are plated in tissue culture dishes and cultured in fibroblast growth medium (FGM) containing: alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 μl/ml).

The fibroblasts are grown and maintained in a humidified atmosphere with 5% CO_2 in air at 37°C.

Adult fibroblast cells are isolated from the lung and skin of a cow. Minced lung tissue is incubated overnight at 10°C in trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). The following day tissue and any disassociated cells are incubated for one hour at 37°C in prewarmed trypsin EDTA solution and processed through three consecutive washes and trypsin incubations (one hr). Fibroblast cells are plated in tissue culture dishes and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 µl/ml). The fibroblast cells can be isolated at virtually any time in development, ranging from approximately post embryonic disc stage through adult life of the animal (bovine day 9 to 10 after fertilization to 5 years of age or longer).

Preparation of fibroblast cells for nuclear transfer

Examples of fetal fibroblasts which may be used as donor nuclei are:

1. Proliferating fibroblast cells that are not synchronized in any one cell stage or serum starved or quiescent can serve as nuclear donors. The cells from the above culture are treated for 10 minutes with trypsin EDTA and are washed three times in 100% fetal calf serum. Single cell fibroblast cells are then placed in micromanipulation drops of HbT medium (Bavister, *et al.*, 1983). This is done 10 to 30 min prior to transfer of the fibroblast cells into the enucleated cow oocyte. Preferably, proliferating transgenic fibroblast cells having the CMV promoter and green fluorescent protein gene (9th passage) are used to produce NT units.

2. By a second method, fibroblast cells are synchronized in G1 or G0 of the cell cycle. The fibroblast cells are grown to confluence. Then the concentration of fetal calf serum in the FGM is cut in half over four consecutive days (day 0 = 10%, day 1 = 5%, day 2 - 2.5%, day 3 = 1.25%, day 4 = 0.625%. On the fifth day the cells are treated for 10 minutes with trypsin EDTA and washed three times in 100% fetal calf serum. Single cell fibroblasts are then placed in micromanipulation drops of

HbT medium. This is done within 15 min prior to transfer of the fibroblast cells into the enucleated cow oocyte.

Removal of cumulus cells

After a maturation period, which ranges from about 30 to 50 hours, and preferably about 40 hours, the oocytes will be enucleated. Prior to enucleation the oocytes will preferably be removed and placed in HECM (Seshagiri and Bavister, 1989) containing 1 milligram per milliliter of hyaluronidase prior to removal of cumulus cells. This may be effected by repeated pipetting through very fine bore pipettes or by vortexing briefly (about 3 minutes). The stripped oocytes are then screened for polar bodies, and the selected metaphase II oocytes, as determined by the presence of polar bodies, are then used for nuclear transfer. Enucleation follows.

EXAMPLE 1

Production of Transgenic Bovine Cultured Inner Cell Mass (CICM) Cells

The defining requirements we used for designating cells as CICM cells were 1) the cells should be derived from the inner cell mass (ICM) of a blastocyst stage

embryo; 2) they should be capable of dividing indefinitely in culture without showing signs of morphological differentiation; and 3) they should contribute to cells of the germ line and endodermal, mesodermal and ectodermal tissues when combined with a host embryo to form a chimera. In addition, cells were evaluated in relation to mouse ES cells for morphology, several cytoplasmic markers and growth characteristics.

Morphologically, the colonies that were established from bovine ICMs maintained distinct margins, had high nuclear to cytoplasmic ratios, generally maintained a high density of lipid granules and were cytokeratin and vimentin negative as in the mouse but, contrary to the mouse, were not positive for alkaline phosphatase. Another difference between mouse cells and bovine CICM cells was that bovine CICM cells were much slower growing than mouse ES cells indicating a much longer cell cycle (estimated to be about 40 hours).

Two methods were used to establish CICM cell colonies from day 7 *in vitro* produced bovine blastocysts. The first method involved isolating the ICM immunosurgically. Anti-sera was developed against bovine spleen cells in mice.

The zona pellucida was removed using 0.5% pronase until the zona thinned and could be removed by pipetting. The blastocysts were exposed to a 1:100 dilution of anti-bovine mouse serum for 45 minutes then washed and treated with guinea pig complement. The lysed trophectodermal cells were removed by pipetting. For the second method, the ICM was isolated mechanically using two 26 gauge needles. The needles were crossed and brought down on the zona intact blastocysts which were cut using a scissors action. Some of the trophectodermal cells remained with the ICM and inevitably disappeared following plating and passaging. A CICM colony was considered established after the third passage without signs of differentiation. For the immunosurgically isolated ICMs 5/9 (55%) formed CICM colonies and for the mechanically isolated ICMs 6/12 (50%) formed colonies. Because no difference was detected between these methods, the mechanical method was adopted for the advantage of simplicity.

Establishment of CICM cell colonies and maintenance of the undifferentiated state depends on an intimate contact between the ICM and the leukemia inhibitory factor producing mouse fibroblast feeder layer. In an attempt to in-

crease the contact during the initial establishment, day 7 *in vitro* produced ICMs were placed either beneath or on top of mouse fetal fibroblast feeder layers. As above, a CICM colony was considered established after the third passage without signs of differentiation. In agreement with previous results 5/9 (55%) ICMs plated on top of the feeder layer produced colonies but only 4/11 (36%) of those placed beneath the feeder layer formed colonies. Apparently, placing the ICMs beneath the feeder layer did not provide the appropriate interaction to inhibit differentiation of the ICMs.

Several methods of passaging bovine CICM cell colonies were attempted. Because it is beneficial to clonally propagate CICM cells following transfection and is necessary for homologous recombination many attempts were made to trypsinize colonies to produce single cells and establish new colonies from these cells. To summarize, all attempts at clonally propagating bovine CICM cells were unsuccessful. Therefore, the routine method of passage that was established was to mechanically cut the colony into pieces that contained at least 50 cells and plate the clumps of cells on new feeder layers.

Following the development of methods of establishing and passaging bovine CICM cells and the identification of limitations in clonally propagating the cells we turned to pursuing methods of transfecting and selecting for transgenic cells. The construct that was used contained a human cytomegalovirus promoter and β-galactosidase/neomycin resistance fusion gene.[12] Selection was based on treatment with Geneticin (G418) to kill nonexpressing cells. The β-galactosidase gene was used to verify incorporation and expression.

Prior to transfecting cells, it was necessary to determine the sensitivity of nontransgenic cells to G418. Colonies from three different embryos were challenged with 0, 50, 100 and 150 $\mu g\ ml^{-1}$ G418. A colony was considered dead when it completely lifted from the feeder layer. Survival varied among lines of cells with the first line surviving an average of 9 days at 100 $\mu m\ ml^{-1}$ and 7 days at 150 $\mu g\ ml^{-1}$. The second line survived 12, 10 and 7 days at 50, 100 and 150 $\mu g\ ml^{-1}$, respectively, and the third line survived 8, 7 and 5 days at 50, 100 and 150 $\mu g\ ml^{-1}$, respectively. To ensure death of all nontransgenic colonies, 150

$\mu g\ ml^{-1}$ G418 was chosen as the dose for subsequent transfection experiments.

Because it was not possible to trypsinize and produce a cell suspension of bovine CICM cells, the method of transfection was limited to either microinjection or lipofection. Various lipofection protocols were tested and found to be effective on fibroblast and Comma D cell cultures but were not effective on bovine CICM cells. Therefore, microinjection was used. CICM cells from three different lines were microinjected into the nucleus with a linearized version of the construct described above. At one day following microinjection, the colonies were treated with 150 $\mu g\ ml^{-1}$ G418 continuously for 30 days. For the three lines 3,753, 3,508 and 3,502 cells were injected and 5, 2 and 0 colonies, respectively, survived selection G418. Some cells within each of these colonies expressed β-galactosidase activity and samples of cells were positive for the transgene when amplified by PCR and analyzed by Southern blot hybridization. Because the colonies essentially disappeared during selection, it is likely that the transgenic lines were of clonal origin, although this was not confirmed. Variation in expression in cells within

a colony was likely due to cell-to-cell variation in factors such as cell cycle state, position effects and others.

Potency of the cells was tested by producing chimeras with host embryos. Prior to evaluating the incorporation of CICM cells into embryos, the relationship between the number of CICM cells injected into morula and the rate of development to the blastocyst stage was investigated. As shown in table 1, either 4, 8 or 12 cells were injected. Rate of development to the blastocyst stage decreased with increasing number of CICM cells used. As an injection control, fibroblasts, either 4, 8 or 12 cells, were injected into morula and as a noninjection control development of a group of nontreated embryos were culture to the blastocyst stage. There were no differences among the numbers of cells injected on development rate, but manipulation, or the injection of cells, did appear to have a detrimental effect on development. Although it was found that increasing the number of CICM cells injected decreased the rate of development, it was also believed that decreasing the number of cells would decrease the level of chimerism in

the embryos. A compromise of injection 8 cells was chosen for further experiments.

Incorporation of CICM cells into bovine blastocysts was evaluated to determine if the CICM cells could interact with the host embryo and be incorporated into the inner cell mass of the blastocyst. CICM cells were labeled with 100 μg ml^{-1} of the fluorescent carbocyanine dye, DiI, then injected into morula stage embryos. Four days later, the resulting blastocysts were observed under the fluorescent microscope. Incorporation of labeled CICM cells into both the ICM and the trophectoderm was detected in all blastocysts. To further verify that the cells were incorporated into the ICM, the trophectoderm was removed by immunosurgery and the isolated ICM was observed. In all cases, labeled cells were detected in the ICM. This indicated that the CICM cells had appropriate cell surface molecules to be incorporated into the compacted morula and ICM and form the early precursors of the fetus.

The next step in examining the potency of the CICM cells was to test chimerism in fetuses recovered at 40 days of gestation. Eighteen day 7 blastocysts, injected with 8 to 10 CICM cells were transferred into six recipient cows.

Forty days after transfer, the fetuses were recovered by Cesarean section. The total number of fetuses recovered was 12 with six being normally developing and 6 dead and in the process of being resorbed. Of the six normal fetuses, the β-GEO transgene was detected in some tissues in all of them (Table 2). Of the abnormal fetuses, it was possible to analyze some tissues in one and it, too, was transgenic. In addition to analyzing somatic tissues, PGCs were isolated and analyzed in the normal fetuses and two showed evidence of transgenic cells. The results of this experiment indicated that the CICM cells did have the capacity to differentiate into many different kinds of tissues, including germ cells, and survive at least 40 days *in vivo*.

Thus, the present invention provides a highly efficient method of producing pluripotent CICM cells in ungulates, or, in particular, for bovines and porcines. Ungulate CICM cells, e.g., bovine or porcine CICM, may be very useful as a source of *in vitro* produced cells for transplantation into humans. Moreover, ungulate cells, e.g., porcine or bovine cells, are potentially useful for gene targeting.

TABLE 1

Effect of Cell Injection on Development of Bovine Morula to the Blastocyst Stage

Type and Number of Cells Injected	Number of Cells Injected Blastocysts (%)	Number of Morula	Blastocyst (%)
ES 15(24)	4	62	15(24)
ES 10(15)	8	65	10(15)
ES 9(13)	12	67	9(13)
Fib 16(30)	4	54	16(30)
Fib 11(19)	8	58	11(19)
Fib 10(28)	12	36	10(28)
Control 19(41)	0	46	19(41)

TABLE 2
Contribution of Transgenic ES Cells to Various Tissues in 40-Day Bovine Fetuses

	Fetus Number					
Tissue	1	2	3	4	5	6
Heart	+	+	-	+	+	+
Muscle	+	-	*	-	*	+
Brain	-	+	+	-	+	+
Liver	*	-	+	-	+	+
Gonads	-	+	+	+	+	+
PGC	+	-	+	-	-	

CICM cell (also contributed to various tissues in the adult animal as shown in Table 4)

Not determined

EXAMPLE 2

Isolation of Primary Cultures of Bovine Fetal and Adult Bovine Fibroblast Cells

Primary cultures of bovine fibroblasts were obtained from fetuses (45 days of pregnancy). The head, liver, heart and alimentary tract were aseptically removed, the fetuses minced and incubated for 30 minutes at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells were plated in tissue culture dishes and cultured in alpha-MEM, medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 µl/ml). The fibroblasts were grown and maintained in a humidified atmosphere with 5% CO_2 in air at 37°C. Cells were passaged regularly upon reaching confluency.

Adult fibroblast cells were isolated from the lung and skin of a cow (approximately five years of age). Minced lung tissue was incubated overnight at 10°C in trypsin EDTA

solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). The following day tissue and any disassociated cells were incubated for one hour at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY) and processed through three consecutive washes and trypsin incubations (one hr). Fibroblast cells were plated in tissue culture dishes and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 µl/ml). The fibroblast cells can be isolated at virtually any time in development, ranging from approximately post embryonic disc stage through adult life of the animal (bovine day 12 to 15 after fertilization to 10 to 15 years of age animals). This procedure can also be used to isolate fibroblasts from other mammals, including mice.

Introduction of a Marker Gene (Foreign Heterologous DNA) Into Embryonic and Adult Fibroblast Cells

The following electroporation procedure was conducted for both fetal and adult bovine fibroblast cells. Standard microinjection procedures may also be used to introduce heterologous DNA into fibroblast cells, however, in this

example electroporation was used because it is an easier procedure.

Culture plates containing propagating fibroblast cells were incubated in trypsin EDTA solution (0.05% trypsin/-0.02% EDTA; GIBCO, Grand Island, NY) until the cells were in a single cell suspension. The cells were spun down at 500 x g and re-suspended at 5 million cells per ml with phosphate buffered saline (PBS).

The reporter gene construct contained the cytomegalovirus promoter and the beta-galactosidase, neomycin phosphotransferase fusion gene (beta-GEO). The reporter gene and the cells at 40 μg/ml final concentration were added to the electroporation chamber. (500 V, ∞ Ohms, 0.4 cm electrode, 250 μF, 500 μL of cell suspension in DPBS) After the electroporation pulse, the fibroblast cells were transferred back into the growth medium (alpha-MEM medium) (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100 IU/ml) and streptomycin (50 μl/ml).

The day after electroporation, attached fibroblast cells were selected for stable integration of the reporter gene. G418 (400 μg/ml) was added to growth medium for 15

days (range: 3 days until the end of the cultured cells' life span). This drug kills any cells without the beta-GEO gene, since they do not express the neo resistance gene. At the end of this time, colonies of stable transgenic cells were present. Each colony was propagated independently of each other. Transgenic fibroblast cells were stained with X-gal to observe expression of beta-galactosidase, and confirmed positive for integration using PCR amplification of the beta-GEO gene and run out on an agarose gel.

Use of Transgenic Fibroblast Cells in Nuclear Transfer Procedures to Create CICM Cell Lines and Transgenic Fetuses

One line of cells (CL-1) derived from one colony of bovine fetal fibroblast cells was used as donor nuclei in the nuclear transfer (NT) procedure. General NT procedures are described above.

Slaughterhouse oocytes were matured *in vitro*. The oocytes were stripped of cumulus cells and enucleated with a beveled micropipette at approximately 18 to 20 hours post maturation (hpm). Enucleation was confirmed in TL-HEPES medium plus Hoechst 33342 (3 μg/ml; Sigma). Individual donor cells (fibroblasts) were then placed in the perivi-

telline space of the recipient oocyte. The bovine oocyte cytoplasm and the donor nucleus (NT unit) were fused together using electrofusion techniques. One fusion pulse consisting of 120 V for 15 μsec in a 500 μm gap chamber filled with fusion medium was applied to the NT unit. This occurred at 24 hpm. The NT units were placed in CR1aa medium until 26 to 27 hpm.

The general procedure used to artificially activate oocytes has been described above. NT unit activation was initiated between 26 and 27 hpm. Briefly, NT units were exposed for four minutes to ionomycin (5 μM; CalBiochem, La Jolla, CA) in TL-HEPES supplemented with 1 mg/ml BSA and then washed for five minutes in TL-HEPES supplemented with 30 mg/ml BSA. Throughout the ionomycin treatment, NT units were also exposed to 2 mM DMAP (Sigma). Following the wash, NT units were then transferred into a microdrop of CR1aa culture medium containing 2 mM DMAP (Sigma) and cultured at 38.5°C and 5% CO_2 for four to five hours. The embryos were washed and then placed in CR1aa medium plus 10% FCS and 6 mg/ml BSA in four well plates containing a confluent feeder layer of mouse embryonic fibroblast. The NT units were cultured for three more days at 38.5°C and 5%

CO_2. Culture medium was changed every three days until days 5 to 8 after activation. At this time blastocyst stage NT embryos can be used to produce transgenic CICM (cultured inner cell mass) cell lines or fetuses. The inner cell mass of these NT units can be isolated and plated on a feeder layer. Also, NT units were transferred into recipient females. The pregnancies were aborted between 35-48 days of gestation. This resulted in seven cloned transgenic fetuses having the beta-GEO gene in all tissues checked. Six of the seven embryos had a normal heart beat detected via ultrasound observation. Also, histological sections of fetuses showed no overt anomalies. Thus, this is a fast and easy method of making transgenic CICM cell lines and fetuses. This procedure is generally conducive to gene targeted CICM cell lines and fetuses.

The table below summarizes the results of these experiments.

TABLE 3

Donor Cell Type	n	Cleavage (%)	Blastocysts (%)	CICM* Lines (%)	Recovered Transgenic Fetuses (%)	Ongoing Pregnancies Past 40 Days
CL-1 bovine fetal fibroblast (bGEO)	412	220 (53%)	40 (10%)	22 (55%)	N/A	N/A
CL-1 bovine fetal fibroblast (bGEO)	3625	2127 (59%)	46 (9%)	N/A	7 fetuses†	9‡
CICM cell line derived from CL-1 NT embryos	709		5 (0.7%)	N/A	0	6Δ
Adult bovine fibroblast	648	331 (51%)	43 (6.6%)	N/A	N/A	1

* 19 lines were positive for beta-GEO, 2 were negative and one line died prior to PCR detection.

† One fetus was dead and another was slightly retarded in development at 35 days of gestation. Five fetuses recovered between 38 to 45 days were normal. All fetuses were confirmed transgenic.

‡ First offspring was born October 1997.

Δ Transgenic chimeric calf born cloned from this line of CICM cells (See Table 4), 6 transgenic chimeric offspring produced.

TABLE 4

	Embryo-derived ES cells				Fibroblast-derived ES cells					
Calf #	901	902	903	904	907	908	909	910	911	912
Skin	-	+	-	-	-	+	-	-	-	-
Muscle	+	-	+	-	+	-	-	-	-	+
Brain	-	-	-	+	-	+	+	+	+	-
Liver	-	-	-	+	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	+	+	+
Kidney	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	+		-	-	-	-	-
Lung	-	-	+	-	-	-	-	-	-	-
Udder	-	+	+	-	-	-	-	-	-	-
Intestine	-	-	+	-	-	-	-	-	-	-
Ovary	na	-	na	na	na	-	na	-	-	-
Testicle	-	na	+	-	-	na	-	na	na	na

EXAMPLE 3

Production of Transgenic Bovine Somatic Cell Nuclear Transplant Embryos

Fibroblasts were chosen as the donor cell because of their ease of isolation, growth and transfection. Bovine fetal fibroblasts were produced from 30 to 100 mm crown rump length (approximately 40 to 80 days of gestation) fetuses obtained from the slaughterhouse. Fetuses were shipped by overnight express mail on ice. In some cases, when a two-day shipment was used, healthy fibroblast lines could still be produced. After propagation for three passages, fibroblasts were transfected by electroporation with a closed circular construct of β-GEO. Following electroporation, transfected cells were selected on 200 μg/ml of G418. After 10 to 15 days on selection, single colonies were isolated, propagated and used for nuclear transfer experiments.

Nuclear transplant blastocysts and fetuses were produced from fibroblasts using standard procedures. Basically, *in vitro* matured oocytes were obtained from Trans Ova Genetics, Inc. by overnight express mail. Oocytes were enucleated using fluorescent labeling of the DNA to verify

enucleation. Trypsinized fibroblast cells were transferred to the perivitelline space and fused to the oocyte cytoplast by electroporation. Activation was induced by a combination of calcium ionophore and 6-dimethylaminopurine. The rate of development to the blastocyst stage was about 10% (353/3625) for nuclear transfer embryos and 14% (106/758) for activated controls. Some blastocysts were shipped to Ultimate Genetics, Inc. for transfer into recipient cows. Two blastocysts were transferred into each recipient. Fetuses recovered at day 40 were morphologically normal and fibroblast cells recovered from these fetuses expressed β-galactosidase at a high level.

Development was allowed to proceed to term in twelve surrogate cows. Seven surrogates gave birth to seven live, vigorous calves, whereas the other five surrogates produced six dead calves or fetuses. Of the seven live calves born, five were delivered by C-section and two by vaginal delivery. One calf was delivered five days before term by c-section after natural labor had begun prematurely and required surfactant. This calf was born from the only cow which did not receive dexmethasone and/or prostaglandin.

Of the six calves who died, one calf died five days after birth; one fetus died in utero seven days before term; one fetus died in utero one month before term; one fetus was aborted one month before term; and two fetuses (twins) died in utero two months before term. Disorders observed in one or more of these cases included hydrallantois, hepatic lipidosis, placental edema of varying severity, and fetal vascular lesions.

The results indicate that fibroblast nuclear transplantation should provide an ideal method of producing transgenic ungulates such as cattle and porcines. Transfection, selection and clonal propagation are relatively easy in primary fibroblasts. The CMV promoter, along with several other constitutive promoters, drive gene expression at a high rate in fibroblasts allowing for routine antibiotic selection. These factors have allowed us to produce a number of transgenic lines with high expressing random gene inserts. Our results also indicate that fibroblasts can be grown for a sufficient number of passages *in vitro*, without going senescent, to allow a second round of selection for a targeted insert. These results suggest that the fibroblast nuclear transplant

system may be a method that will finally allow the commercial production of transgenic livestock for improved agricultural production.

EXAMPLE 4

Bovine Chimeric Offspring Produced by Transgenic CICM Cells Generated From Somatic Cell Nuclear Transfer Embryos

Genetic modifications of bovine CICMs, particularly targeted integrations, would be of use for the production of transgenic cattle or for the production of *in vitro* derived tissues for transplantation into humans. Previous work in our laboratory indicated that bovine CICM are slow growing and cannot be clonally propagated; limiting their usefulness for direct genetic modification. Therefore, an alternate approach for genetically modifying bovine CICMs was investigated. Somatic cells have been used in the past to generate bovine blastocysts (Collas and Barnes, *Mol. Reprod. Devel.*, 38:264-267; 1994) and may be used to produce CICM cells. In this study, fetal fibroblasts were transfected then fused with enucleated oocytes to generate blastocysts and, subsequently, transgenic CICM cells. The

potency of these CICM cells was then tested by their ability to form chimeric calves.

Fetal bovine fibroblasts were isolated from a 60 day fetus. Cells were stably transfected by electroporation with a cytomegalovirus promoter and a β-galactosidase/-neomycin resistance fusion gene (β-Geo). After three weeks of negative cell selection on 400 μg/ml of Geneticin (Sigma, St. Louis, MO), single transgenic colonies were isolated and determined positive for β-galactosidase activity and PCR analysis. Fibroblasts were grown on 150 μg/ml of Geneticin and, upon reaching 70 to 80% confluency, used for nuclear transplantation. Enucleated *in vitro* matured bovine oocytes were fused with actively dividing fibroblasts and chemically activated by ionomycin and 6-dimethylaminopurine. Following activation, embryos were cultured for 3 days in CR2 (Specialty Media, Lavallette, NJ) with 1% fetal calf serum (FCS; HyClone, Logan, UT) and mouse embryonic fibroblasts (MEF) as a co-culture, from day 4 to the blastocyst stage, embryos were cultured with 10% FCS. Thirty-seven nuclear transfer blastocysts out of 330 (11%) were produced and plated in MEF, 22 (60%) of those generated CICM cell lines. Morphologically, these CICM

cells were similar to those described earlier (Cibelli et al, *Therio.*, 47:241; 1997), i.e., high nuclear/cytoplasmic ratio, the presence of lipid bodies and several nucleoli. In order to test the pluripotency of these cells *in vivo*, eight to ten transgenic CICM cells were injected into 8-16 cell bovine embryos. A total of 99 chimeric embryos were produced, 22 (22%) of them reached blastocyst stage and 10 of those were transferred into five recipient cows. Six calves were born (60%) and, upon ear sample screening by PCR amplification and Southern blot hybridization of the amplified product to a β-galactosidase fragment, one calf was detected positive (17%). In situ DNA hybridization indicated that about 30% of the cells in the spleen were derived from the CICM cells in this calf. Also, the CICM cells contributed to cells within the testes.

This work demonstrates that ungulate somatic cells can be dedifferentiated and CICM cells produced, opening the possibility of using them, not only for the generation of transgenic ungulates, in particular porcines and bovines, but, also, in differentiation studies and cell therapy.

EXAMPLE 5

Expression of Exogenous DNA by Cloned Transgenic Cattle

Fibroblasts from female Holstein fetuses are established in culture using the methods described above. Cells are plated at a concentration of 2-3 X 10^6 cells/ml in 100mm well plates and cultured with alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% FCS, 100 IU/ml penicillin and 50 μl/ml streptomycin. The plates are incubated at 37°C with 5% CO_2. The media is changed every 3 days, and cells passaged regularly upon reaching confluency.

Culture plates sufficient to provide approximately 100,000 propagating fibroblast cells are incubated with trypsin-EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY) until the cells are in a single cell suspension. The cells are spun-down at 500 xg and resuspended to a concentration of 10^6-10^7 cells/ml in PBS with potassium concentrations greater than 400 μg/ml.

The reporter gene is a human serum albumin-neomycin (hSA-neo) linearized gene construct.

Approximately 50 to 100 μg of the DNA construct is added to the isolated fibroblast cell suspension. The

cells and DNA are placed in an electroporation chamber and pulsed with 300-500 V. After the electroporation pulse, the fibroblast cells are transferred back into the growth medium (alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), 100 IU/ml penicillin and 50 μl/ml streptomycin).

Selection for stable integration of the construct into the fibroblast cells is done over the next 5 to 15 days using G418 (400 μg/ml) as described above. The presence of the construct is confirmed by Southern blot analysis in surviving cell colonies. The cell lines may also be karyotyped to check for aneuploidy and polyploidy. Surviving transgenic fibroblast colonies are clonally propagated in the presence of greater than 5% serum and are actively propagating.

Cell lines with the construct stably integrated are used for nuclear transfer procedures. General nuclear transfer procedures are described above.

Female cattle are induced to superovulate with an injection of GNRH. Approximately 20 to 24 hours after GNRH injection the *in vivo* matured oocytes are collected from

the ovaries and oviducts of the donor females. The expanded cumulus cells are stripped from the oocytes and the MII chromosomes removed from the oocytes via micromanipulation.

Three to five clonal transgenic fibroblast cell lines are used in the nuclear transfer procedure. Clonal transgenic fibroblasts are incubated with a trypsin/EDTA solution, spun-down, and resuspended in fusion medium. Individual transgenic fibroblasts are placed in the perivitelline space of the recipient enucleated oocyte.

Individual transgenic fibroblast cells are fused with an enucleated oocyte in fusion media using electrofusion to produce a fused NT unit. One fusion pulse consisting of 120V for 15 μsec in a 500 μm gap chamber filled with fusion medium is applied to the chamber. This occurs at 24 hours past maturation (hpm). The fused NT units are placed in TL-HEPES medium for 15-30 minutes to allow the fusion to proceed.

The fused NT units are placed in B_2 culture media a balanced salt solution that does not contain calcium lactate. The B_2 medium contains a protein kinase inhibitor to initiate oocyte activation, thus preventing the fused NT units from forming chromosomes.

An hour after initiation of activation, the NT units are exposed to 5 μM ionomycin for 4 minutes. The fused NT units are washed and resuspended in B_2 medium plus a protein kinase inhibitor (6-dimethylamino purine) for three hours. After incubation with the protein kinase inhibitor, the fused NT units are placed into B_2 medium without a protein kinase inhibitor and co-cultured with mouse fibroblasts cells or buffalo rat liver (BRL) cells.

The fused NT units are cultured to the blastocyst stage and nonsurgically transferred into a synchronized recipient female animal with 1-2 embryos per recipient. Pregnancies are monitored by ultrasound at 40, 60, and 90 days gestation. Confirmed transgenic offspring are maintained under specified good agricultural practices and herd health programs. The level of expression of hSA in their milk is confirmed over a 30-day period (approximately 2 months after induced lactation).

EXAMPLE 6

Transgenic Bovine Neurons Produced by Somatic Cell Cloning for Transplantation in Parkinsonian Rats

Mesencephalic tissue from 42 to 50 day-old cloned transgenic bovine fetuses was tested for survival and effect on disease after being transplanted into the striata of hemiparkinsonian rats. Fetal bovine fibroblasts derived by enzymatic digestion from a bovine fetus (50 mm crown rump length) were used as donors of nuclei for the nuclear transfer. Prior to the nuclear transfer, *lacZ* and neomycin resistance genes were stably transfected into the fetal bovine fibroblasts by electroporation. The construct CMV/βgeo (Acc#J95-34) was used. Neomycin resistant cells were selected by incubation with G418 for 15 days.

The transfected cells were used as donors of genetic material to efficiently produce transgenic cloned fetuses. Donor fibroblasts used in the nuclear transfer were actively dividing as evidenced by positive immunocytochemistry to proliferating cell nuclear antigen (PCNA).

After oocytes were obtained from the slaughterhouse and matured *in vitro*, they were stripped of cumulus cells and enucleated with a beveled pipette. Enucleation of the

oocytes was confirmed using Hoechst 33342 DNA dye. Individual donor fibroblasts were placed next to the perivitelline space of the recipient oocyte. The two cells were fused by a 90 volt electrical pulse lasting for 14 μsec.

The nuclear transfer resulted in 8% of the embryos forming blastocysts (**Table 5**). In control parthenogenetically activated oocytes, 13% of the embryos formed blastocysts. After 7 or 8 days in culture the resulting blastocysts were transferred into recipient females. The implantation resulted in 38% pregnancies developing past 40 days.

TABLE 5

Efficiency of nuclear transfer to produce blastocysts using fetal bovine fibroblasts as donors of genetic material.

type of oocyte	n	cleavage	blastocyst
parthenogenetically activated oocytes (control)	61	40(66%)	8(13%)
nuclear transfer oocytes (transgenic clone)	414	267(64%)	34(8%)

Cloned bovine fetuses were detected by ultrasound and aborted between 42 and 50 days of gestation. Average crown

rump length for the wild type fetuses was 19.9 ± 1.5 mm and 17.3 ± 3.2 mm for the cloned fetuses (Fig. 1A). All of the cloned fetuses produced were genetically identical and transgenic. Fibroblasts derived from these fetuses expressed in the β-galactosidase transgene as assayed by X-gal staining (Fig. 1B).

Ventral mesencephalon was dissected as previously described (31). PCT analysis was performed to verify the presence of the *lacZ* gene as follows. DNA was extracted from a strand of cloned transgenic ½ mesencephalon cultured for 7 days using a Q1Aamp Tissue Kit (Qiagen). DNA contained in the transplant tract hemisphere and the contralateral to the transplant hemisphere was extracted from the 40 μm brain sections as previously described. (Shedlock et al, *BioTechniques*, 22:394-399 (1997)) PCR reaction underwent 30 cycles using a pair of primers (5'-CGCTGTGGTACACGCTGTGCG-3' and 5'-TCCCCAGCGACCAGATGATCGC-3'), and ^{32}P-labeled PCR products were detected on a phospho-imager (BioRad). This analysis revealed the presence of *lacZ* gene in a mesencephalon cultured for one week and in the transplanted cloned mesencephalon (Fig. 1C). The

transgene however was not found in the side of the brain contralateral to the transplant, in the transplants of the wild type mesencephalon and in the transplants of the vehicle (Fig. 1C).

To test survival of dopamine neurons and β-galactosidase expression *in vitro*, primary cultures of bovine ventral mesencephalon were prepared in 1 ml of ice cold Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (Mediatech) by mechanically dispersing tissue pieces using a sterile tip of a 1.0 ml Pipetman as previously described. Subsequently, cells were centrifuged at 200xg for 5 min and resuspended in F12 medium (Irvine Sci.) with 5% human placental serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 2.2 μg/ml ascorbic acid. Cells were seeded at a density was 6.0×10^4 viable cells/cm^2 in polyethylenimine (Sigma) coated 96-well plates in 0.1 ml of media. Cells were incubated in a 95% air/5% CO_2 humidified atmosphere at 37°C. 50% of medium was changed every third day.

Dopamine neurons were identified by immunocytochemistry for tyrosine hydroxylase (TH) (63) as illustrated in Figure 2. Bovine dopamine neurons survived in culture for

at least 12 days. Between days 2 and 12 in culture, the number of surviving wild type dopamine neurons decreased by 71% from 1185 ± 88 to 343 ± 38 per cm^2 (Fig. 2C). During the same period of time, the number of surviving cloned dopamine neurons decreased by 81% from 2325 ± 94 to 322 ± 65 per cm^2 (Fig. 2C). We and others have previously observed similar death rates of dopamine neurons in primary cultures of rat and human mesencephalon (55). β-galactosidase was expressed for at least 12 days *in vitro* as revealed by immunocytochemistry using a polyclonal antibody (Fig. 2A, B) (1:500, 5'-3', Boulder, CO).

To test if bovine mesencephalic tissue produced dopamine, dissected mesencephalon was cultured as tissue strands for a week and the culture media was assayed for the presence of homovanillic acid (HVA), a stable metabolite of dopamine, by high pressure liquid chromatography as previously described (63). Tissue strands (200 μm in diameter) were created by extruding ½ (for tissue culture) or ¼ (for transplantation) of mesencephalon through a tapered glass cannula made by heating a commercially available blank (Kimble Kontes, Cat# 663500-0444). Wild type mesencephalic strands (n=6) produced on average 5.4 ±

0.5 pmoles of HVA per day. Similarly, a strand of a cloned mesencephalon produced 7.3 pmoles of HVA per day.

After demonstrating that cloned mesencephalic tissue yields viable dopamine producing neurons, the bovine neurons were transplanted into parkinsonian rats. Hemiparkinsonian rats received transplants of ¼ of a bovine ventral mesencephalon or infusion of vehicle (Ca^2/Mg^{2+}-free Hanks' balanced salt solution) into the deneravated striatum (AP: 0.0 mm form bregma, LAT: 3.0 mm form the midline, VD: -3.5 to -7.5 mm below the dura) in 4.0 μl over 4 min. All transplanted rats were immunosuppressed 24 hrs prior to transplantation with Cyclosporine A (Sandimmune; 10 mg/kg; sc; Sandoz) and daily thereafter for the duration of the experiment.

The unilateral 6-OHDA lesions of the nigrostriatal pathway in these rats were created at least four weeks prior to transplantation. Twenty male Sprague-Dawley rats (225-250 gm) were anesthetized with equithesin (4ml/kg) and placed in a stereotaxic frame. Lesions of the medial forebrain bundle of the left hemisphere were done by infusing 20 μg of 6-OHDA HBr (RBI), dissolved in 4 μl of sterile saline containing 0.2% ascorbate at 1 μl/min per

site at 2 sites (AP: -2.1 mm posterior to bregma, LAT: 2.0 mm from the midline, VD: -7.8 mm below the dura; and AP: -4.3 mm posterior to bregma, LAT: 1.5 mm from the midline, VD: -7.8 mm below the dura).

The dopaminergic deficit was demonstrated in lesioned animals by rotational asymmetry in response to injection of 5.0 mg/kg methamphetamine. Animals were tested for response to methamphetamine (5.0 mg/kg) two weeks after receiving lesions and assigned to groups of equal rotational rates: 1. -vehicle, (n-5, RPM=9.0±1.5): 2. -clone (n=8, RPM=8.5±1.2); 3. -wild type (n=7, RPM=8.5±1.0).

One month after transplantation, the rotational rate of animals transplanted with cloned mesencephalon was reduced to 58 ± 15% of the pretransplant rate (Fig. 3A). The rotational rate in animals receiving wild type mesencephalic tissue was also reduced to 70 ± 35% of the pretransplant value. By contrast, animals that received vehicle (Ca^2/Mg^{2+} free Hanks' balanced salt solution) did not show any behavioral improvement and their rotational rate was maintained at 97 ± 9% of the pretransplant value.

The behavioral improvement in animals transplanted with cloned tissue was even more apparent at two months after transplantation when the rotational rate was further reduced to 52 ± 16% of the pretransplant value (Fig. 3A). The overall reduction in the circling rate of the animals receiving cloned tissue was statistically significant when compared with vehicle controls $F_{1,17}=8.0, p<0.05$).

At two months after transplantation, animals receiving wild type mesencephalic tissue lost the motor benefits provided by the graft. This may have been due to the activation of the immune response observed in animals from both cloned (Fig. 4) and wild type groups. At two months, the animals receiving vehicle continued to circle at 107 ± 23% of the pretransplant rate.

After sacrifice, 603 ± 246 surviving dopamine neurons were identified by TH immunocytochemistry in transplants of the cloned mesencephalon (64). Graft-containing areas of each brain were sectioned in the coronal plane at 40 μm thickness and mounted on glass microscope slides. Every sixth slide was stained for TH-immunoreactivity using a polyclonal antibody against rat TH (Pel-Freez) and ABC straining kit (Vector). Following deparaffinization,

endogenous peroxidase was inactivated by a 20 min treatment in methanol containing 20% hydrogen peroxide (v/v) at room temperature. Nonspecific binding was blocked with 10% goat serum in PBS containing 1% BSA and 0.3% Triton-X for 60 min at room temperature. After rinsing with PBS, a primary rabbit-anti-rat-TH antibody (1:100 dilution) was applied to each slide overnight at 37°C. Sections were then incubated with a biotinylated, affinity-purified, goat anti-rabbit IgG antibody and subsequently with avidin/biotinylated horseradish peroxidase complex, each for 2 hrs at room temperature. The peroxidase was visualized with diamino-benzidine dissolved in PBS and 0.03% hydrogen peroxide. All TH-positive profiles were counted in each section. Abercrombie's correction assumed cell diameter of 20 μm and was used to generate the final estimate of the number of surviving dopamine neurons in each animal.

Animals transplanted with wild type mesencephalic tissue had 956 ± 416 surviving dopamine neurons. Dopamine neurons were not observed in any of the vehicle transplants. Non-linear regression (Fig. 3B) revealed that the number of surviving dopamine neurons correlated with the improvement in the motor behavior (r^2=0.565). Overall,

two months after transplantation, about 1000 dopamine neurons were required to reduce the rotational behavior in response to methamphetamine by at least 50%. Surviving dopamine cells spanned large areas of the striatum (Fig. 5A, C) and projected neurites into the host brain (Fig. 5B, D). Animals receiving vehicle transplants did not yield any dopamine neurons in the transplant tracts (Fig. 5E).

Our observations show that cloned bovine embryonic dopamine cells can survive transplantation into brain and improve behavior in a rat model of Parkinson's disease. This model has predicted the success of human fetal tissue survival in human Parkinson's disease patients and thus provides strong evidence that cloned ungulate cells, such as cloned bovine or porcine cells, may prove useful for treatment of human Parkinsonism.

Because the genetic makeup of all cells contributing to the somatically cloned embryos used in these experiments was identical, it resulted in a better characterized and more stable phenotype. Cloned transgenic bovine fetal dopamine cells survived transplantation and produced significant reduction in rotational behavior in Parkinsonian rats. It is expected that similar or even better

results will be achieved using cloned porcine fetal dopamine cells.

Our estimate of the number of dopamine cells required to reduce circling by 50% in response to methamphetamine is higher than that obtained from xenotransplantation of pig dopamine neurons into Parkinsonian rats (53). This is likely a result of a shorter experimental course used in our experiments (2 months) as compared with the pig xenotransplantation study that lasted for 4 months allowing for more complete development of the transplanted neurons.

Introduction of additional genes and/or do gene targeting in fibroblast derived cloned fetuses is a fast and efficient method of producing genetically manipulated fetal tissue for transplantation. Since xenografts attract lymphocytic infiltration, introduction of genes encoding peptides with immunosuppressant properties into the cloned tissue could reduce the chance of rejection. Introduction of genes encoding human growth factors that are neurotrophic to dopamine neurons could further improve survival of the transplants and enhance behavioral recovery.

Our results demonstrate for the first time that fetal tissue produced by somatic cell cloning can be used in treatment of a neurodegenerative disease.

EXAMPLE 7

Recloning in Bovine

Ungulate cells, and more specifically bovine or porcine cells used as nuclear donors, have a finite life span. Even more specifically, the fetal bovine fibroblasts which are preferably used for nuclear transfer procedures have a limited life span. When cultured until senescence, fibroblasts derived from 6 weeks old bovine fetuses undergo approximately 30 population doublings (PD) and have a cell cycle length of 28 to 30 hr. While this PD is adequate to generate clonally derived transgenic cell lines, it may be inadequate to achieve multiple gene targeting events wherein two or more rounds of selection must be performed. It may be inadequate because cells may become senescent before the desired genetic modifications are effected. Given these circumstances, the present inventors have compared population doubling of fibroblasts derived from a non-manipulated fetus and a nuclear transfer fetus. The PD

were 31.36 and 32.64 respectively. This data suggests that the fibroblast's life span can be enhanced by nuclear transfer procedures. Moreover, it indicates that the present approach can be repeated to generate as many gene targeting events as needed by subjecting the cell line to successive rounds of transfection, selection, nuclear transfer, fetus production and fibroblast isolation. This "recloning" procedure as it is called is depicted schematically in Figure 6. Essentially, this procedure comprises the production of a cloned, transgenic ungulate embryos, e.g., a cloned bovine or porcine embryo, the cells of which are then isolated, manipulated *in vitro* to introduce another genetic modification, e.g., targeted deletion or addition, and the resultant cells used as nuclear donors to produce another cloned, transgenic NT embryo. This embryo will comprise the genetic modifications introduced in both cloning procedures. Moreover, based on the observed PDs for non-manipulated versus NT fetus, recloning can be repeated as many times as necessary to introduce the desired deletions and/or insertions.